No. 200300043

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

Coors Brewing Company

Whereas, THERE HAS BEEN PRESENTED TO THE

Secretary of Agriculture

AN APPLICATION REQUESTING A CERTIFICATE OF PROTECTION FOR AN ALLEGED DISTINCT VARIETY OF SEXUALLY REPRODUCED, OR TUBER PROPAGATED PLANT, THE NAME AND DESCRIPTION OF WHICH ARE CONTAINED IN THE APPLICATION AND EXHIBITS, A COPY OF WHICH IS HEREUNTO ANNEXED AND MADE A PART HEREOF, AND THE VARIOUS REQUIREMENTS OF LAW IN SUCH CASES MADE AND PROVIDED HAVE BEEN COMPLIED WITH, AND THE TITLE THERETO IS, FROM THE RECORDS OF THE PLANT VARIETY PROTECTION OFFICE, IN THE APPLICANT(S) INDICATED IN THE SAID COPY, AND WHEREAS, UPON DUE EXAMINATION MADE, THE SAID APPLICANT(S) IS (ARE) ADJUDGED TO BE ENTITLED TO A CERTIFICATE OF PLANT VARIETY PROTECTION UNDER THE LAW.

NOW, THEREFORE, THIS CERTIFICATE OF PLANT VARIETY PROTECTION IS TO GRANT UNTO THE SAID APPLICANT(S) AND THE SUCCESSORS, HEIRS OR ASSIGNS OF THE SAID APPLICANT(S) FOR THE TERM OF TWENTY YEARS FROM THE DATE OF THIS GRANT, SUBJECT TO THE PAYMENT OF THE REQUIRED FEES AND PERIODIC REPLENISHMENT OF VIABLE BASIC SEED OF THE VARIETY IN A PUBLIC REPOSITORY AS PROVIDED BY LAW, THE RIGHT TO EXCLUDE OTHERS FROM SELLING THE VARIETY, OR OFFERING IT FOR SALE, OR REPRODUCING IT, OR IMPORTING IT, OR EXPORTING IT, OR CONDITIONING IT FOR PROPAGATION, OR STOCKING IT FOR ANY OF THE ABOVE PURPOSE , OR USING IT IN PRODUCING A HYBRID OR DIFFERENT VARIETY THEREFROM, TO THE EXTENT PROVIDED BY THE PLANT VARIETY PROTECTION ACT. IN THE UNITED STATES SEED OF THIS VARIETY (1) SHALL BE SOLD BY VARIETY NAME ONLY AS A CLASS OF CERTIFIED SEED AND (2) SHALL CONFORM TO THE NUMBER OF GENERATIONS SPECIFIED BY THE OWNER OF THE VARIETY. (84 STAT. 1542, AS AMENDED, 7 U.S.C. 2321 ET SEQ.)

BARLEY

'IdaGold II'

In Testimony Whereof, *I have hereunto set my hand and caused the seal of the Plant Variety Protection Office to be affixed at the City of Washington, D.C. this sixteenth day of September, in the year two thousand three.*

Attest:

Commissioner
Plant Variety Protection Office
Agricultural Marketing Service

Secretary of Agriculture

REPRODUCE LOCALLY. ***Include form number and date on all reproductions*** Form Approved - OMB No. 0581-0055

U.S. DEPARTMENT OF AGRICULTURE
AGRICULTURAL MARKETING SERVICE
SCIENCE AND TECHNOLOGY - PLANT VARIETY PROTECTION OFFICE

APPLICATION FOR PLANT VARIETY PROTECTION CERTIFICATE

(Instructions and information collection burden statement on reverse)

The following statements are made in accordance with the Privacy Act of 1974 (5 U.S.C. 552a) and the Paperwork Reduction Act (PRA) of 1995.

Application is required in order to determine if a plant variety protection certificate is to be issued (7 U.S.C. 2421). Information is held confidential until certificate is issued (7 U.S.C. 2426).

1. NAME OF OWNER
Coors Brewing Company

2. TEMPORARY DESIGNATION OR EXPERIMENTAL NAME
C32

3. VARIETY NAME
IdaGold II

4. ADDRESS *(Street and No., or R.F.D. No., City, State, and ZIP Code, and Country)*
12th and Ford Street
Golden, Colorado 80401
United States of America

5. TELEPHONE *(include area code)*
(303) 279-6565

6. FAX *(include area code)*
(303) 277-7373

FOR OFFICIAL USE ONLY

PVPO NUMBER
200300043

FILING DATE
11-25-2002

7. IF THE OWNER NAMED IS NOT A "PERSON", GIVE FORM OF ORGANIZATION *(corporation, partnership, association, etc.)*
Corporation

8. IF INCORPORATED, GIVE STATE OF INCORPORATION
Colorado

9. DATE OF INCORPORATION
June 12, 1913

10. NAME AND ADDRESS OF OWNER REPRESENTATIVE(S) TO SERVE IN THIS APPLICATION. *(First person listed will receive all papers)*
Jay K. Malkin
KLAAS, LAW, O'MEARA & MALKIN, P.C.
1999 Broadway, Suite 2225
Denver, Colorado 80202
United States of America

FEES RECEIVED

FILING AND EXAMINATION FEES:
$ 2705.00
DATE

CERTIFICATION FEE:
$ 432.00
DATE 4/9/2003

11. TELEPHONE *(include area code)*
(303) 298-9888

12. FAX *(include area code)*
(303) 297-2266

13. E-MAIL
KLAASLAW@ATT.NET

14. CROP KIND *(Common Name)*
2-Row Spring Feed Barley

15. GENUS AND SPECIES NAME OF CROP
Hordeum vulgare L.

16. FAMILY NAME *(Botanical)*
Poaceae (Gramineae)

17. IS THE VARIETY A FIRST GENERATION HYBRID?
☐ YES ☑ NO

18. CHECK APPROPRIATE BOX FOR EACH ATTACHMENT SUBMITTED (Follow instructions on reverse)

- a. ☑ Exhibit A. Origin and Breeding History of the Variety
- b. ☑ Exhibit B. Statement of Distinctness
- c. ☑ Exhibit C. Objective Description of Variety
- d. ☐ Exhibit D. Additional Description of the Variety *(Optional)*
- e. ☑ Exhibit E. Statement of the Basis of the Owner's Ownership
- f. ☑ Voucher Sample *(2,500 viable untreated seeds or, for tuber propagated varieties, verification that tissue culture will be deposited and maintained in an approved public repository)*
- g. ☑ Filing and Examination Fee ($2,705), made payable to "Treasurer of the United States" *(Mail to the Plant Variety Protection Office)*

19. DOES THE OWNER SPECIFY THAT SEED OF THIS VARIETY BE SOLD AS A CLASS OF CERTIFIED SEED? *See Section 83(a) of the Plant Variety Protection Act)*
☑ YES *(If "yes", answer items 20 and 21 below)* ☐ NO *(If "no", go to item 22)*

20. DOES THE OWNER SPECIFY THAT SEED OF THIS VARIETY BE LIMITED AS TO NUMBER OF CLASSES? ☐ YES ☑ NO
IF YES, WHICH CLASSES? ☐ FOUNDATION ☐ REGISTERED ☐ CERTIFIED

21. DOES THE OWNER SPECIFY THAT SEED OF THIS VARIETY BE LIMITED AS TO NUMBER OF GENERATIONS? ☐ YES ☑ NO
IF YES, SPECIFY THE NUMBER 1,2,3, *etc.* ☐ FOUNDATION ☐ REGISTERED ☐ CERTIFIED
(If additional explanation is necessary, please use the space indicated on the reverse.)

22. HAS THE VARIETY (INCLUDING ANY HARVESTED MATERIAL) OR A HYBRID PRODUCED FROM THIS VARIETY BEEN SOLD, DISPOSED OF, TRANSFERRED, OR USED IN THE U. S. OR OTHER COUNTRIES?
☑ YES ☐ NO
IF YES, YOU MUST PROVIDE THE DATE OF FIRST SALE, DISPOSITION, TRANSFER, OR USE FOR EACH COUNTRY AND THE CIRCUMSTANCES. *(Please use space indicated on reverse.)*

23. IS THE VARIETY OR ANY COMPONENT OF THE VARIETY PROTECTED BY INTELLECTUAL PROPERTY RIGHT *(PLANT BREEDER'S RIGHT OR PATENT)*?
☐ YES ☑ NO
IF YES, PLEASE GIVE COUNTRY, DATE OF FILING OR ISSUANCE AND ASSIGNED REFERENCE NUMBER. *(Please use space indicated on reverse.)*

24. The owners declare that a viable sample of basic seed of the variety will be furnished with application and will be replenished upon request in accordance with such regulations as may be applicable, or for a tuber propagated variety a tissue culture will be deposited in a public repository and maintained for the duration of the certificate.

The undersigned owner(s) is(are) the owner of this sexually reproduced or tuber propagated plant variety, and believe(s) that the variety is new, distinct, uniform, and stable as required in Section 42, and is entitled to protection under the provisions of Section 42 of the Plant Variety Protection Act.

Owner(s) is(are) informed that false representation herein can jeopardize protection and result in penalties.

SIGNATURE OF OWNER
Coors Brewing Company Hugo Patino

NAME *(Please print or type)*
Hugo Patino

CAPACITY OR TITLE
Vice President - Quality, Research, & Development

DATE
Nov. 22, 2002

SIGNATURE OF OWNER

NAME *(Please print or type)*

CAPACITY OR TITLE

DATE

INSTRUCTIONS

2003 00043

GENERAL: To be effectively filed with the Plant Variety Protection Office (PVPO), **ALL** of the following items must be **received** in the PVPO: (1) Completed application form signed by the owner; (2) completed exhibits A, B, C, E; (3) for a seed reproduced variety at least 2,500 viable untreated seeds, for a hybrid variety at least 2,500 untreated seeds of each line necessary to **reproduce** the variety, or for tuber reproduced varieties verification that a viable *(in the sense that it will reproduce an entire plant)* tissue culture will be deposited and maintained in an approved public repository; (4) check drawn on a U.S. bank for $2,705 ($320 filing fee and $2,385 examination fee), payable to "Treasurer of the United States" *(See Section 97.6 of the Regulations and Rules of Practice.)* Partial applications will be held in the PVPO for not more than 90 days, then returned to the applicant as unfiled. Mail application and other requirements to Plant Variety Protection Office, AMS, USDA, Room 401, NAL Building, 10301 Baltimore Avenue, Beltsville, MD 20705-2351. Retain one copy for your files. All items on the face of the application are self explanatory unless noted below. Corrections on the application form and exhibits must be initialed and dated. **DO NOT** use masking materials to make corrections. If a certificate is allowed, you will be requested to send a check payable to "Treasurer of the United States" in the amount of $320 for issuance of the certificate. Certificates will be issued to owner, not licensee or agent.

Plant Variety Protection Office
Telephone: (301) 504-5518
FAX: (301) 504-5291
Homepage: http://www.ams.usda.gov/science/pvpo/pvp.htm

ITEM

18a. Give:
(1) the genealogy, including public and commercial varieties, lines, or clones used, and the breeding method;
(2) the details of subsequent stages of selection and multiplication;
(3) evidence of uniformity and stability; and
(4) the type and frequency of variants during reproduction and multiplication and state how these variants may be identified

18b. Give a summary of the variety's distinctness. Clearly state how this application variety may be distinguished from all other varieties in the same crop. If the new variety is most similar to one variety or a group of related varieties:

(1) identify these varieties and state all differences objectively;
(2) attach statistical data for characters expressed numerically and demonstrate that these are clear differences; and
(3) submit, if helpful, seed and plant specimens or photographs (prints) of seed and plant comparisons which clearly indicate distinctness.

18c. Exhibit C forms are available from the PVPO Office for most crops; specify crop kind. Fill in Exhibit C (Objective Description of Variety) form as completely as possible to describe your variety.

18d. Optional additional characteristics and/or photographs. Describe any additional characteristics that cannot be accurately conveyed in Exhibit C. Use comparative varieties as is necessary to reveal more accurately the characteristics that are difficult to describe, such as plant habit, plant color, disease resistance, *etc.*

18e. Section 52(5) of the Act requires applicants to furnish a statement of the basis of the applicant's ownership. An Exhibit E form is available from the PVPO.

19. If "Yes" is specified *(seed of this variety be sold by variety name only, as a class of certified seed)*, the applicant **MAY NOT** reverse this affirmative decision after the variety has been sold and so labeled, the decision published, or the certificate issued. However, if "No" has been specified, the applicant may change the choice. *(See Regulations and Rules of Practice, Section 97.103).*

22. *See Sections 41, 42, and 43 of the Act and Section 97.5 of the regulations for eligibility requirements.*

23. *See Section 55 of the Act for instructions on claiming the benefit of an earlier filing date.*

21. CONTINUED FROM FRONT *(Please provide a statement as to the limitation and sequence of generations that may be certified.)*

~~22. CONTINUED FROM FRONT~~ ~~*(Please provide the date of first sale, disposition, transfer, or use for each country and the circumstances, if the variety (including any harvested material) or a hybrid produced from this variety has been sold, disposed of, transferred, or used in the U.S. or other countries.)*~~

No commercial activities (e.g. sales or offers for sale) or exploitations have been made to date regarding IdaGold II. All activities undertaken thus far involving IdaGold II were entirely and exclusively done for testing, experimentation and/or increase purposes as per 7 USC 2401(b). Exhibits A and B (incorporated herein by reference) specifically disclose activities pertaining to the completion of IdaGold II including all applicable dates, countries, and locations associated with these activities.

23. CONTINUED FROM FRONT *(Please give the country, date of filing or issuance, and assigned reference number, if the variety or any component of the variety is protected by intellectual property right (Plant Breeder's Right or Patent).)*

NOTES: It is the responsibility of the applicant/owner to keep the PVPO informed of any changes of address or change of ownership or assignment or owner's representative during the life of the application/certificate. There is no charge for filing a change of address. The fee for filing a change of ownership or assignment or any modification of owner's name is specified in Section 97.175 of the regulations. *(See Section 101 of the Act, and Sections 97.130, 97.131, 97.175(h) of the Regulations and Rules of Practice.)*

To avoid conflict with other variety names in use, the applicant must check the appropriate recognized authority. For example, for agricultural and vegetable crops, contact: Seed Branch, AMS, USDA, Room 213, Building 306, Beltsville Agricultural Research Center--East, Beltsville, MD 20705. Telephone: (301) 504-8089. http://www.ams.usda.gov/lsg/seed.htm

S&T-470 (07-01) designed by the Plant Variety Protection Office with WordPerfect 9.0. Replaces STD-470 (04-01) which is obsolete.

EXHIBIT A

200300043

Origin and Breeding History of IdaGold II

1. **GENEALOGY/BREEDING PEDIGREE:**

(A) Cross breeding and selection of the claimed variety (previously identified by temporary experimental number "C32" but now designated hereinafter as "IdaGold II" which shall be considered the final variety name thereof) was performed by James M. Jakicic, Roy J. Hanson, Kathy R. Adams, Berry J. Treat, Dennis J. Dolan, and James W. Hettinger beginning with parental barley plantings in late fall of 1991 at the Coors Brewing Company (Owner) Malting Barley Research Center, Burley ID (USA). All of the foregoing individuals were employees of the Coors Brewing Company during their work on the development of IdaGold II.

(B) The origin of IdaGold II is as follows:

MH 3/12/2003 per correspondence of 2/26/03

i. C19 = Triumph / Crystal /// Triumph / Harrington // Nairn ~~Crystal [female] was crossed with IdaGold [male] (e.g. Crystal [female] x IdaGold [male]) to yield a further product designated herein for informational purposes as "C19".~~ It should be noted that, for the purposes of this and the other Exhibits in the current application, the name "IdaGold" should be interpreted to involve the first IdaGold variety, namely, IdaGold I compared with the second IdaGold variety which is the subject of the current application (e.g. IdaGold II).

ii. Grit [female] was crossed with Andrea [male] (e.g. Grit [female] x Andrea [male]) to yield a still further product designated herein for informational purposes as "C13".

iii. The product of the cross set forth above in breeding sequence [i] (e.g. C19) was then crossed as a female with the product of the cross set forth above in breeding sequence [ii] (e.g. C13) as a male (e.g. C19 [female] x C13 [male]) to

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yield IdaGold II (formerly "C32" as previously stated).

In summary, the entire breeding sequence is as follows:

"Triumph/Crystal/3/Triumph/Harrington//Nairn/4/Grit/Andrea" ~~"Crystal/IdaGold//Grit/Andrea"~~ or (expressed in a more short-hand form): **"C19//C13"**.

MH 3/12/03 per correspondence of 2/26/2003

(C) Background information regarding the above-listed parental varieties is as follows:

i. Crystal - This variety was developed by the USDA/ARS, Aberdeen, ID (USA) [Dr. D. Wesenberg]. It was specifically obtained from the following cross: "Columbo/Klages". The resulting product was designated as "78AB6871" prior to release as Crystal.

ii. IdaGold - The Coors Brewing Company developed this variety, with its pedigree being: "Moravian III/Summit//Triumph".

iii. C19 - The Coors Brewing Company also developed this variety, with its pedigree being: "Triumph/Crystal///Triumph/Harrington//Nairn".

iv. Grit - Developed in 1983 in Berlin, Germany. Cooperators = Veb Zaat- und Pflanzgut with its pedigree being: "Hadmerslebener 5547/Emir/11191/Union/46459/Diamant/14008". (Source of information: USDA-ARS "GRIN" database).

v. Andrea - The pedigree of this variety is proprietary and unavailable.

vi. C13 - The Coors Brewing Company likewise developed this variety, with its pedigree being: "Grit/Andrea".

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2. **DETAILS OF SUBSEQUENT STAGES OF SELECTION AND MULTIPLICATION**

MAH 3/12/2003

(A) The basic cross listed above (e.g. ~~"Crystal/IdaGold//Grit/Andrea" or~~ "C19//C13" [as previously stated]) was undertaken by the Coors Brewing Company Malting Barley Research Station at its greenhouse located in Burley, ID (USA) during the late fall of 1991 and extending into 1992.

(B) F1 seeds from the above-listed basic cross (designated as "91-28") were harvested and increased by planting in the greenhouse at the Coors Brewing Company Malting Barley Research Station in Burley, ID (USA) during the winter/spring of 1992.

(C) F2 bulked seeds were then planted in the field at the Coors Brewing Company Malting Barley Research Station in Burley, ID (USA) in the spring of 1992 for segregating plant selection. The plants chosen for subsequent development were single head selected and harvested.

(D) F3 and F4 single plants were grown using a single seed descent method in the greenhouse at the Coors Brewing Company Malting Barley Research Station in Burley, ID (USA) during the fall/winter of 1992 and extending into 1993. The F4 plants were single head selected and harvested.

(E) F5 head rows were planted in the field at the Coors Brewing Company Malting Barley Research Station in Burley, ID (USA) in the spring of 1993. Superior rows were identified and head selections were made at harvest.

(F) F6 individual head selections were then increased in a three (3)-meter row in New Zealand during the winter of 1993 and extending into 1994. The row was bulk harvested as pure seed.

(G) F7 Observation trial plots were planted at the Coors Brewing Company Malting Barley Research Station in Burley, ID (USA) during the summer of 1994. The resulting line was identified as superior and renamed "C91-286". C91-286 was selected for advancement based on its expression of superior agronomic and malting characteristics, as well as high yield and very large, plump seed.

(H) F8 C91-286 was planted in replicated/multiple location yield trials in the spring/summer of 1995 at the Coors Brewing Company Malting Barley Research Station in Burley, ID (USA) and at other locations in the USA. Based on agronomic test data, C91-286 was chosen for advanced breeder seed development. At harvest, (F9) multiple head selections were taken for subsequent breeders' seed head rows.

(I) F9 head selections were increased in New Zealand during the winter of 1995 and extending into 1996.

(J) F10 breeders' seed was planted in the summer of 1996 at the Coors Brewing Company Malting Barley Research Station in Burley, ID (USA). At that time, C91-286 was renamed and given the experimental designation "Coors 32" or simply "C32".

(K) F11 foundation seed of C32 was increased in 1997 at the Coors Brewing Company Malting Barley Research Station in Burley, ID (USA). Thereafter, C32 entered large production scale seed increases. In 2001, C32 was renamed "IdaGold II".

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3. **EVIDENCE OF UNIFORMITY AND STABILITY:**

Variety IdaGold II has been reproduced and judged stable for the past six (6) generations. Variety IdaGold II is uniform for all traits as described in Exhibit C (Objective Description of the Variety). Variety IdaGold II shows no variants other than what would normally be expected due to environment.

4. **SELECTION CRITERIA:**

To assist in the examination and assessment of this application, specific information will now be provided regarding the selection criteria associated with IdaGold II. Such criteria were generally based on agronomic performance, with the specific selection criteria of primary concern being as follows: (1) high grain yield; (2) plant height; (3) lodging resistance; (4) disease resistance; (5) plant phenotype; (6) kernel discoloration; (7) test weight; (8) plump kernels; (9) protein levels; and (10) early maturity. Additional information concerning the breeding of IdaGold II is provided above in this Exhibit, with the distinguishing and differentiating characteristics of IdaGold II relative to its parents being set forth in Exhibit B.

EXHIBIT B - STATEMENT OF DISTINCTNESS

This Exhibit will be divided into two sections. The first section involves genetic data which definitively distinguishes the claimed barley variety (IdaGold II) from its parental varieties (including the two varieties that are closest to IdaGold II, namely, Crystal and IdaGold [e.g. IdaGold I]). Additional information regarding Crystal and IdaGold is presented in Exhibit C which is part of the current application.

The second section of the present Exhibit discloses a significant amount of agronomic data including but not limited to information involving differences in Plant Height, Color, Plot Yield, and other characteristics as explained below. Again, this data provides clear and convincing support for a finding of distinctness regarding IdaGold II compared with the other varieties listed herein (including but not limited to Crystal and IdaGold).

Either the genetic data or the agronomic data (when considered separately from each other) will support the allowance of this application. However, even further support for this position is provided by a **combination** of the genetic and agronomic data which offers clear, convincing, and undeniable evidence of the distinctness of IdaGold II over the next closest varieties and the other varieties discussed below.

A. Genetic Data

A complex and detailed genetic testing protocol was undertaken in connection with IdaGold II, as well as the parental varieties associated therewith (e.g. Andrea, Crystal, Grit, IdaGold, C13, and C19). It is again noted that Crystal and IdaGold are considered to be the closest varieties to IdaGold II as noted in, for instance, Exhibit C. However, as will be extensively discussed below, the genetic tests outlined in this section provide definitive evidence that IdaGold II is entirely distinct and unique relative to the other varieties listed in this Exhibit (including Crystal and IdaGold).

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At this point, the genetic testing protocol (along with some background information involving the testing procedures that were employed and the scientific theories behind the tests) will now be provided. The genetic tests in this case were conducted by STA Laboratories, Inc. (1821 Vista View Drive, Longmont, CO [USA] 80504; Telephone: 1-303-651-6417; Internet: www.stalabs.com) which is highly experienced in testing processes of the type outlined herein. Likewise, the following explanation of the testing procedures that were used, background information and text associated therewith, data tables, and photographs were generally provided by STA Laboratories which is hereby acknowledged.

1. BACKGROUND

As will be discussed extensively in this section of Exhibit B, a DNA analysis was conducted on seven (7) barley varieties (namely, Andrea, Crystal, Grit, IdaGold [e.g. IdaGold I], C13, C19, and IdaGold II). It should be noted that the terms "C32" and "IdaGold II" are used interchangeably and equivalently throughout this discussion, with both terms involving the same barley variety. C32 was the temporary/experimental name for the barley variety being claimed in this application which was later changed to IdaGold II (See the historical discussion presented in Exhibit A).

The DNA analysis that was conducted in this case involved "Polymerase Chain Reaction" (e.g. "PCR") technology which enabled a unique DNA "fingerprint" to be obtained for each of the aforementioned varieties. In particular, seeds associated with the above-listed barley varieties were initially germinated (in the dark) for a five (5) day period. Two (2) sets of four (4) seedlings were selected for DNA extraction and analysis. DNA extractions were performed using a "modified CTAB-based method" as discussed in, for example, Khasa, P.D., et al., "Isolation, Characterization, and Inheritance of Microsatellite Loci in Alpine Larch and Western Larch", Genome, 43:439 - 448 (2000). A copy of this article is attached hereto for reference purposes as Attachment B-1. PCR (e.g. "Polymerase Chain Reaction") analysis was then

performed using both anchored and non-anchored ISSR primers (discussed further below). The resulting PCR products were subsequently separated on horizontally-oriented 5% acrylamide gel structures which were thereafter stained with silver. The gel structures were then dried and digitized.

Having presented a brief explanation of the testing procedures that were employed, some basic background information will now be provided concerning the technology and theories associated with the analytical methods of interest. If the reader of this Exhibit is already familiar with this information, he or she may skip this section and proceed to the next section which discusses specific test results involving IdaGold II.

A significant majority of the DNA in a cell is comprised of various amino acid sequences which do not contain any genetic information (also known as "non-coding DNA"). For many years, the function of non-coding DNA has been considered from a theoretical point of view. Dispersed throughout this DNA are simple amino acid sequences which are repeated many times. These sequences are traditionally known as "microsatellites" or "SSRs" (namely, "simple sequence repeats" or "short sequence repeats"). These sequences vary in length and composition. A variety of examples are provided below with the understanding that the number of possible alternatives are virtually unlimited. Some representative microsatellites are as follows:

1. **ATATATATATATATATATAT**

2. **CTTCTTCTTCTTCTTCTTCTT**

3. **GTGTGTGTGTGTGTGTGTGT**

[wherein A = adenine; T = thymine; and G = guanine]

Because these microsatellite structures are scattered throughout an organism's entire genome, they can be isolated, analyzed, and ultimately employed as genetic "markers" in many useful applications. In particular, by

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determining the DNA sequences adjacent to a microsatellite sequence, it is possible to design a "primer sequence" which can be employed in PCR experiments to determine the presence or absence of the particular microsatellite in a chosen plant variety. For example, consider the following primer sequence:

CGCTATTCCGTATC

The presence of this primer sequence in the following chain can be used to detect the existence of a given microsatellite therein (e.g. GGTGGTGGTGGTGGTGGTGGT) which is shown in italics as follows:

...CGCTATTCCGTATC*GGTGGTGGTGGTGGTGGTGGT*...
(microsatellite)

For example, a particular microsatellite may be linked very tightly to a gene which controls "fruit size" in pumpkins. Thus, when a pumpkin plant yields a pumpkin having a weight exceeding 100 lbs., the microsatellite may, in fact, be found in the DNA of the plant 97% of the time, thus making microsatellite identification an important tool in plant identification and differentiation. This technology can also be used by a plant breeder to cross two inbred lines, with the breeder thereafter looking for the microsatellite sequence of interest in the progeny. By adopting this procedure, many benefits can be achieved including but not limited to a considerable saving in the time needed to accurately characterize the progeny. For instance, in the pumpkin example listed above, the plant breeder would not have to wait for a full growing season to be completed in order to determine what size pumpkins will be produced. Instead, the breeder could do immediate genetic tests to see if the microsatellite for "pumpkin size" is in the pumpkin plants before the pumpkins are fully grown. Microsatellite markers can also be employed to measure the purity of a hybrid seed line to be certain

that the seed does not contain a large quantity of the inbred lines which were used to produce it.

The development and generation of microsatellite markers as discussed above is normally an expensive and labor-intensive process. Specifically, a considerable amount of time, effort, and expense is needed in order to isolate and characterize the desired sequences, synthesize the primers, and test the markers for utility and effectiveness. A method which enables the use of microsatellite sequences as markers but does not require characterization of the "flanking" sequences is known as "Inter-Simple Sequence Repeats" or "ISSRs". In accordance with this technique, a primer is synthesized which contains a portion of the actual repeated sequence. At one end of the primer is a sequence of three (3) nucleotides which is used to "anchor" the primer to the three (3) nucleotides adjacent the repeated sequence. Since the actual adjacent sequence is not known, the primers are designed so that they contain a mixture of different nucleotides in the "terminal 3" positions. As a result, it is likely that some of the primers in the mixture will have exactly the correct sequence. This type of primer is known as an "anchored" ISSR.

It is also possible to use primers which bind only to the repeated sequence, itself. These particular primers are known as "non-anchored" ISSR primers which are further discussed in Bornet, B., et al., "Nonanchored Inter Simple Sequence Repeat (ISSR) Markers: Reproducible and Specific Tools for Genome Fingerprinting", Plant Molecular Biology Reporter, 19:209 - 215 (2001) - (copy attached as Attachment B-2). An example of such a situation is as follows:

(primers)
AAGGGTGGTGGTGGTGGT
TTCGGTGGTGGTGGTGGT
TAGGGTGGTGGTGGTGGT
***ATC*GGTGGTGGTGGTGGT**
CGCTATTCCGT*ATC*GGTGGTGGTGGTGGTGGTGGT....
(microsatellite)

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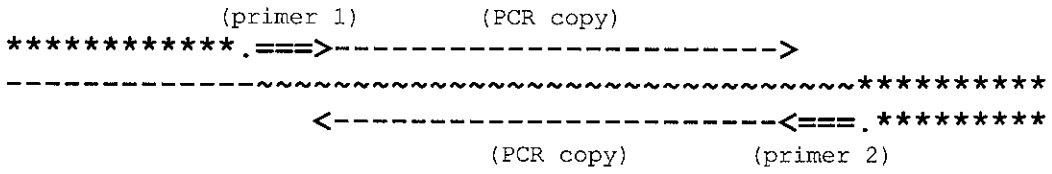
Furthermore, when two primers bind in opposite directions and are reasonably close together, the PCR process can be used to copy the DNA sequences between primers as follows:



A "colorized" representation of the above-listed sequencing configuration is attached hereto as Attachment B-3 for reference purposes. As a result of the foregoing situation, a DNA fragment of a specific length is created. The above-listed process typically leads to many PCR products/fragments, each with a specific length. This "set" of DNA fragments serves as a "fingerprint" which can be employed as a unique "identifier" for a particular crop species and/or variety which is highly accurate.

Having set forth general background information involving the technology of concern, the following more detailed and specific PCR data is provided along with photographs which clearly and definitively distinguish IdaGold II from all of the above-mentioned varieties including but not limited to Crystal and IdaGold (the two closest varieties as recited in Exhibit C).

2. Test Data

*****POINT OF INFORMATION NO. 1*****

The data discussed in this Section of Exhibit B involves a comprehensive study entitled, "Cultivar Differentiation in Barley Using ISSR PCR". As noted above, "ISSR" stands for "Inter-Simple Sequence Repeats", with "PCR" meaning "Polymerase Chain Reaction". Regarding the study disclosed herein, the following barley varieties were tested and compared: (1) Andrea; (2) Crystal; (3) Grit; (4)

IdaGold [namely, IdaGold I]; (5) C13; (6) C19; and (7) C32 (which is another name for IdaGold II as previously indicated).

As shown in the photograph of Attachment B-4, barley variety C32/IdaGold II is clearly distinguished from Andrea, Crystal, Grit, IdaGold, and C13 (with Crystal and IdaGold again being considered the closest varieties to IdaGold II as per the information provided in Exhibit C). Specifically (and with continued reference to Attachment B-4), one PCR product was found between 123 bp and 246 bp (in particular, at approximately 200 bp) which is significantly different in C19 and C32/IdaGold II compared with all of the other varieties. The term "bp" as used herein is an abbreviation for "base pairs". **Accordingly and as stated above, both of these varieties (e.g. C19 and C32/IdaGold II) are clearly distinguished from the other five (5), with C32/IdaGold II therefore being entirely distinct relative to Andrea, Crystal, Grit, IdaGold, and C13.** The PCR product discussed in this paragraph which is used to distinguish C19 and C32/IdaGold II from the other varieties (Andrea, Crystal, Grit, IdaGold, and C13) and vice versa is identified in Attachment B-4 at the blue-green arrow. It should likewise be noted that the primer associated with the test protocol of Attachment B-4 involved a "non-anchored 5X CAG".

Further test data will be provided below which clearly distinguishes C19 from C32/IdaGold II, thereby confirming that C32/IdaGold II is unique and distinct compared with **all** of the varieties being studied including the ones closest thereto (Crystal and IdaGold).

*****POINT OF INFORMATION NO. 2*****

As illustrated in the photograph of Attachment B-5, barley variety C32/IdaGold II is clearly distinguished from C19 (as well as C13). Specifically (and with continued reference to Attachment B-5), a PCR product was found between 369 bp and 861 bp (in particular, at approximately 831 bp) which is significantly different in C32/IdaGold compared with C19 and C13. **Accordingly and as stated above, C32/IdaGold II is clearly distinguished from C19 and C13 and is therefore entirely distinct relative thereto.** The

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PCR product discussed in this paragraph which is used to distinguish C32/IdaGold II from the other varieties mentioned herein (C19 and C13) and vice versa is identified in Attachment B-5 at the blue-green arrow.

It should likewise be noted that the primer associated with the test protocol of Attachment B-5 involved a "non-anchored 4X GACA".

Thus, at this point, definitive genetic evidence has been provided regarding the distinctness of C32/IdaGold II compared with **all** of the other varieties mentioned above (Andrea, Crystal, Grit, IdaGold, C13, and C19). The additional data set forth below involves a comparison between the foregoing parental varieties (not C32/IdaGold II) and is being provided for academic and informational purposes.

*****POINT OF INFORMATION NO. 3*****

As shown in the photograph of Attachment B-4, barley variety C19 is clearly distinguished from Andrea, Crystal, Grit, and IdaGold. Specifically (and with continued reference to Attachment B-4), a PCR product was found between 123 bp and 246 bp (in particular, at approximately 200 bp) which is significantly different for C19 compared with Andrea, Crystal, Grit, and IdaGold. The PCR product discussed in this paragraph which is used to distinguish C19 from all of the above-mentioned varieties (Andrea, Crystal, Grit, and IdaGold) and vice versa is identified in Attachment B-4 at the blue-green arrow.

In accordance with Attachment B-5, barley variety C19 is clearly distinguished from C13. Specifically (and with continued reference to Attachment B-5), a PCR product was found between 369 bp and 861 bp (in particular, at approximately 831 bp) which is significantly different for C19 compared with C13. The PCR product discussed in this paragraph which is used to distinguish C19 from C13 and vice versa is identified in Attachment B-5 at the blue-green arrow.

POINT OF INFORMATION NO. 4

As shown in the photograph of Attachment B-6, barley variety Grit (and C13 which was indicated to be identical to Grit in this particular portion of the test) is clearly distinguished from Andrea. Specifically (and with reference to Attachment B-6), a PCR product was found between 1107 bp and 1353 bp (in particular, at approximately 1230 bp) which is significantly different for Grit (and C13) compared with Andrea. The PCR product discussed in this paragraph which is used to distinguish Grit (and C13) from Andrea (and vice versa) is identified in Attachment B-6 at the top blue-green arrow.

With continued reference to the photograph of Attachment B-6, barley variety Grit (and C13 as noted above) is clearly distinguished from Crystal based on a PCR product which was found between 246 bp and 369 bp (in particular, at approximately 300 bp). This PCR product is significantly different for Grit (and C13) compared with Crystal. The PCR product discussed in this paragraph which is used to distinguish Grit (and C13) from Crystal (and vice versa) is identified in Attachment B-6 at the bottom blue-green arrow.

It should likewise be noted that the primer associated with the test protocol of Attachment B-6 involved "(TCC)5RY".

POINT OF INFORMATION NO. 5

As shown in the photograph of Attachment B-7, barley variety Crystal is clearly distinguished from Andrea. Specifically (and with continued reference to Attachment B-7), a PCR product was found at approximately 615 bp which is significantly different for Crystal compared with Andrea. The PCR product discussed in this paragraph which is used to distinguish Crystal from Andrea (and vice versa) is identified in Attachment B-7 at the blue-green arrow.

In addition, the primer associated with the test protocol of Attachment B-7 involved "HVH(TG)7T".

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POINT OF INFORMATION NO. 6

As shown in the photograph of Attachment B-8, barley variety IdaGold (e.g. IdaGold I) is clearly distinguished from Andrea. Specifically (and with continued reference to Attachment B-8), a PCR product was found at approximately 738 bp which is significantly different for IdaGold compared with Andrea. The PCR product discussed in this paragraph which is used to distinguish IdaGold from Andrea (and vice versa) is identified in Attachment B-8 at the blue-green arrow.

It should likewise be noted that the primer associated with the test protocol of Attachment B-8 involved a mixture of "(TCC)5RY" and "HVH(TG)7T".

**********DATA SUMMARY**********

Attachment B-9 involves a summary of the information provided above regarding the significant genetic differences which exist between C32/IdaGold II and all of the other listed varieties. Basically and with particular reference to the last horizontal line in the Table of Exhibit B-9 (marked with an "*"), it is indicated that C32/IdaGold II is entirely distinct compared with Andrea, Crystal, Grit, IdaGold (namely, IdaGold I), C13, and C19. Each of the "intersecting" boxes in Exhibit B-9 involves (1) the particular primer under consideration; and (2) the approximate size of the "key" DNA band in base pairs (bp). In this regard, the following summary of information in the Table of Exhibit B-9 is highly relevant:

1. **C32/IdaGold II v. Andrea** -

(i) Primer: No. 1 ("non-anchored 5X CAG");
(ii) Key DNA band size: 200 bp;
(iii) Attachment which shows this information: B-4; and
(iv) **Conclusion**: C32/IdaGold II is clearly distinct compared with Andrea.

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2. **C32/IdaGold II v. Crystal (**one of the two closest varieties to C32/IdaGold II**)** -

(i) Primer: No. 1 ("non-anchored 5X CAG");
(ii) Key DNA band size: 200 bp;
(iii) Attachment which shows this information: B-4; and
(iv) **Conclusion**: C32/IdaGold II is clearly distinct compared with Crystal.

3. **C32/IdaGold II v. Grit** -

(i) Primer: No. 1 ("non-anchored 5X CAG");
(ii) Key DNA band size: 200 bp;
(iii) Attachment which shows this information: B-4; and
(iv) **Conclusion**: C32/IdaGold II is clearly distinct compared with Grit.

4. **C32/IdaGold II v. IdaGold (**e.g. IdaGold I which is one of the two closest varieties to C32/IdaGold II**)** -

(i) Primer: No. 1 ("non-anchored 5X CAG");
(ii) Key DNA band size: 200 bp;
(iii) Attachment which shows this information: B-4; and
(iv) **Conclusion**: C32/IdaGold II is clearly distinct compared with IdaGold.

5. **C32/IdaGold II v. C13** -

(i) Primers: No. 1 ("non-anchored 5X CAG") and No. 4 ("4x GACA");
(ii) Key DNA band sizes: 200 bp and 831 bp;
(iii) Attachments which show this information: B-4 and B-5; and
(iv) **Conclusion**: C32/IdaGold II is clearly distinct compared with C13.

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6. **C32/IdaGold II v. C19** -

(i) Primer: No. 4 ("4X GACA");
(ii) Key DNA band size: 831 bp;
(iii) Attachment which shows this information: B-5; and
(iv) **Conclusion**: C32/IdaGold II is clearly distinct compared with C19.

As can be seen from the above-mentioned information, the genetic data set forth in this Exhibit provides clear, convincing, and definitive evidence of the distinctness of IdaGold II. It is therefore believed that this information, by itself, is sufficient to support the allowance of the current application. Thus, the analysis could stop at this point. However, agronomic data will now be presented which provides even further support for the approval of this case. The agronomic data listed below could likewise stand alone as evidence of distinctness regarding IdaGold II as will become clear from the following discussion. Accordingly, when both the genetic and agronomic data listed in this Exhibit are considered **collectively**, they provide undeniable support for the novelty and uniqueness of IdaGold II.

B. Agronomic Data

Agronomic data was collected and reviewed on the barley varieties associated with the pedigree involving variety IdaGold II (namely, the parents of IdaGold II). This data clearly distinguishes IdaGold II from the above-mentioned varieties (including those varieties which are considered to be the most similar to and/or which most closely resemble IdaGold II). These closest varieties include (1) Crystal; and (2) IdaGold (e.g. IdaGold I) as is likewise recited in Exhibit C which accompanies this application.

The tests discussed below were designed to compare IdaGold II with the parental varieties set forth throughout the current application. The objective of the testing

processes was to provide an unbiased appraisal and evaluation of IdaGold II from a comparative agronomic standpoint. Basically, the tests summarize trials conducted by the Coors Brewing Company (Applicant/Owner) in multiple years and locations including (1) Burley, ID (USA); (2) Berthoud, CO (USA); and (3) Center, CO (USA).

Virtually all of the test data provided below includes a "Least Significant Difference" (LSD) statistic. Where "LSD" statistics are given, they are presented at the 0.05% error level and are an aid in comparing varieties. The Coefficient of Variation ("CV" or "COV") statistic is also included in most of the data summaries/tables and provides a general measure of the precision associated with each experimental trial. For the record and reflective of the information presented herein, all ANOVA's were run with "Fisher's Pairwise Comparisons" at a 0.05% individual error rate. Furthermore, the present variety of interest for which protection is sought will be identified in the Data Tables discussed below as "C32" or "IdaGold II" which are the same and shall thus be considered equivalent terms. As previously stated, C32 was the temporary/experimental name for the barley variety being claimed in this application which was later changed to IdaGold II (See the historical discussion presented in Exhibit A).

There are **clear and substantial differences** in important genetic, morphological, and phenotypical characteristics when IdaGold II is compared with the listed parental varieties (including but not restricted to Crystal and IdaGold). These differences overwhelmingly support the allowability of the present application under all applicable statutory guidelines. The data of interest and primary concern will now be discussed as follows which further supplements the genetic information provided at the beginning of Exhibit B (and clearly supports the novelty of IdaGold II as expressed herein).

Furthermore, the test results discussed below are of particular relevance in connection with Crystal and IdaGold (e.g. IdaGold I) which are considered to be the closest varieties relative to IdaGold II as previously stated. In particular, the following test data (along with the genetic information recited at the beginning of this Exhibit) clearly demonstrates that IdaGold II is distinctive relative to Crystal, IdaGold, and the other listed varieties with reference to **multiple characteristics** and

not just a single item. This multiplicity of differences (alone or combined with the genetic data set forth herein) overwhelmingly supports a determination that IdaGold II is entitled to plant variety protection over Crystal, IdaGold, Andrea, Grit, C13, C19, and any other barley varieties.

In order to assist in assessing the data and comparisons presented below (with particular reference to the Data Tables associated with this Exhibit), the following definitions are applicable (which are standard, conventional, and well-known in this technical field):

A. **Plot Yld.** = "Plot Yield" = The actual amount of barley seed (in lbs.) that is obtained from a single plot which is approximately 4 ft. x 20 ft;

B. **Test Wt.** = "Test Weight" = A weight-by-unit volume measurement involving the barley seed being tested which is initially measured in Grams/Dry Quart and then converted using a test weight scale into a test weight value which is equal to Lbs/Bu (Pounds/Bushel);

C. **Bu/Ac** = "Bushels per Acre";

D. **Lbs/Ac @ 12% H_2O** = "Pounds per acre at 12% water" = A corrected value used to represent the pounds of barley seed at 12% moisture. This value is used so that all varieties from a given trial can be compared since the moisture level varies significantly by variety and can have a substantial impact on weight. The formula used for this calculation is as follows: (Lbs/Plot x 820.6 x [1 - % Moisture of Sample (see definition below) x 0.01]) x 1.142857143;

E. **% Screen over 6/64** = A value referring to the % of seed remaining from a 100 gram seed sample after being run on a shaker for 20 cycles using a 6/64 inch screen;

F. **Lbs/Ac over 6/64** = A corrected value used to represent the Lbs/Ac (Pounds/Acre) of "plump" seed in a given sample. The term "plump" as used in connection with the barley seed is defined to involve the particular seed which are associated with the % Screen over 6/64 value

(defined above) which remain from a 100 gram seed sample after being run on a shaker for 20 cycles using a 6/64 inch screen. The corrected value associated with the Lbs/Ac over 6/64 parameter is obtained using the value determined for Lbs/Ac @ 12% H_2O (see definition above). In particular, the formula used for this calculation is as follows: (Lbs/Ac @ 12% H_2O x % Screen Over 6/64 [see definition above] x 0.01);

G. **Moisture** = A value involving the % moisture remaining in the harvested barley kernel (seed). It was determined in the current assessment using a Perten 9100 NIR Whole Grain Analyzer (Perten Instruments Inc. USA, 6444 South 6th Street Road, Springfield IL 62707);

H. **Protein** = A value involving the % protein present in the harvested barley kernel (seed). It was determined in the current assessment using a Perten 9100 NIR Whole Grain Analyzer;

I. **Color** = A value on a scale from 0 - 100 with 0 being the darkest and 100 being the brightest kernel (seed) color possible. It was determined in the current assessment using a Perten 9100 NIR Whole Grain Analyzer;

J. **Height** = Plant Height of the barley plants in inches;

K. **Lodging** = The percent of the plot area that was not standing straight prior to harvest; and

L. **Days to Head** = "Heading Date" = The number of days from planting (or other specified date) that it takes for 50% of the heads to emerge from the boot.

1. **IdaGold II v. IdaGold (one of the two varieties closest to IdaGold II as previously stated.)**

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As noted above in the first portion of this Exhibit which pertains to comparative genetic data, both IdaGold II and IdaGold (e.g. IdaGold I) are characterized by distinctively different genetic profiles. This data constitutes definitive evidence of the unique nature of IdaGold II relative to IdaGold and is sufficient, by itself, to support the allowability of IdaGold II as a totally distinct variety compared with IdaGold. However, as supplemental/extra evidence, the following agronomic differences and data are provided concerning IdaGold II and IdaGold:

[i] **IdaGold II has a significantly earlier Heading Date compared with IdaGold.**

This key difference is clearly illustrated in:

A. The Data Table provided herewith as Attachment B-10 (entitled "1996 SIVPT~Southern Idaho Variety Performance Trial, Burley ID"). This Table sets forth a Heading Date of **77.25** for IdaGold II v. a Heading Date of **97** for IdaGold.

B. The Data Table provided herewith as Attachment B-11 (entitled "1996 NCVPT~Northern Colorado Variety Performance Trial, Berthoud CO"). This Table sets forth a Heading Date of **88.5** for IdaGold II v. a Heading Date of **93.75** for IdaGold.

C. The Data Table provided herewith as Attachment B-12 (entitled "1996 SCVPT~Southern Colorado Variety Performance Trial, Center CO"). This Table sets forth a Heading Date of **78.5** for IdaGold II v. a Heading Date of **80.5** for IdaGold.

D. The Data Table provided herewith as Attachment B-13 (entitled "2001 PVP~Plant Variety Protection Trial, Burley ID"). This Table sets forth a Heading Date of **77.6667** for IdaGold II v. a Heading Date of **78.6667** for IdaGold.

[ii] **IdaGold II has a significantly higher Plot Yield compared with IdaGold.**

This key difference is clearly illustrated in:

A. The Data Table provided herewith as Attachment B-10 (entitled "1996 SIVPT~Southern Idaho Variety Performance Trial, Burley ID"). This Table sets forth a Plot Yield value of **11.935** for IdaGold II v. a Plot Yield value of **10.31** for IdaGold.

B. The Data Table provided herewith as Attachment B-12 (entitled "1996 SCVPT~Southern Colorado Variety Performance Trial, Center CO"). This Table sets forth a Plot Yield value of **9.825** for IdaGold II v. a Plot Yield value of **8.35** for IdaGold.

C. The Data Table provided herewith as Attachment B-11 (entitled "1996 NCVPT~Northern Colorado Variety Performance Trial, Berthoud CO"). This Table sets forth a Plot Yield value of **8.8** for IdaGold II v. a Plot Yield value of **7.645** for IdaGold.

D. The Data Table provided herewith as Attachment B-13 (entitled "2001 PVP~Plant Variety Protection Trial, Burley ID"). This Table sets forth a Plot Yield value of **9.13333** for IdaGold II v. a Plot Yield value of **8.52** for IdaGold.

[iii] **IdaGold II yields significantly greater Bushels/Acre (Bu/Ac) compared with IdaGold.**

This key difference is clearly illustrated in:

A. The Data Table provided herewith as Attachment B-10 (entitled "1996 SIVPT~Southern Idaho Variety Performance Trial, Burley ID"). This table sets forth a Bu/Ac value of **200.543** for IdaGold II v. a Bu/Ac value of **173.251** for IdaGold.

B. The Data Table provided herewith as Attachment B-12 (entitled "1996 SCVPT~Southern Colorado Variety

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Performance Trial, Center CO"). This table sets forth a Bu/Ac value of **163.949** for IdaGold II v. a Bu/Ac value of **139.551** for IdaGold.

C. The Data Table provided herewith as Attachment B-11 (entitled "1996 NCVPT~Northern Colorado Variety Performance Trial, Berthoud CO"). This table sets forth a Bu/Ac value of **147.904** for IdaGold II v. a Bu/Ac value of **129.053** for IdaGold.

D. The Data Table provided herewith as Attachment B-13 (entitled "2001 PVP~Plant Variety Protection Trial, Burley ID"). This table sets forth a Bu/Ac value of **150.761** for IdaGold II v. a Bu/Ac value of **141.577** for IdaGold.

[iv] **IdaGold II has a significantly higher Pounds per Acre at 12% Water value compared with IdaGold.**

This key difference is clearly illustrated in:

A. The Data Table provided herewith as Attachment B-10 (entitled "1996 SIVPT~Southern Idaho Variety Performance Trial, Burley ID"). This Table sets forth a Pounds per Acre at 12% Water value of **10027.2** for IdaGold II v. a Pounds per Acre at 12% Water value of **8662.6** for IdaGold.

B. The Data Table provided herewith as Attachment B-12 (entitled "1996 SCVPT~Southern Colorado Variety Performance Trial, Center CO"). This Table sets forth a Pounds per Acre at 12% Water value of **8197.47** for IdaGold II v. a Pounds per Acre at 12% Water value of **6977.56** for IdaGold.

C. The Data Table provided herewith as Attachment B-11 (entitled "1996 NCVPT~Northern Colorado Variety Performance Trial, Berthoud CO"). This Table sets forth a Pounds per Acre at 12% Water value of **7395.2** for IdaGold II v. a Pounds per Acre at 12% Water value of **6452.65** for IdaGold.

D. The Data Table provided herewith as Attachment B-13 (entitled "2001 PVP~Plant Variety Protection Trial,

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Burley ID"). This Table sets forth a Pounds per Acre at 12% Water value of **7538.07** for IdaGold II v. a Pounds per Acre at 12% Water value of **7078.84** for IdaGold.

[v] **IdaGold II has a significantly higher Pounds per Acre over 6/64 value compared with IdaGold.**

This key difference is clearly illustrated in:

A. The Data Table provided herewith as Attachment B-11 (entitled "1996 NCVPT~Northern Colorado Variety Performance Trial, Berthoud CO"). This Table sets forth a Pounds per acre over 6/64 value of **6346.81** for IdaGold II v. a Pounds per Acre over 6/64 value of **3733.01** for IdaGold.

B. The Data Table provided herewith as Attachment B-10 (entitled "1996 SIVPT~Southern Idaho Variety Performance Trial, Burley ID"). This Table sets forth a Pounds per Acre over 6/64 value of **9497.46** for IdaGold II v. a Pounds per Acre over 6/64 value of **8054.16** for IdaGold.

C. The Data Table provided herewith as Attachment B-12 (entitled "1996 SCVPT~Southern Colorado Variety Performance Trial, Center CO"). This Table sets forth a Pounds per Acre over 6/64 value of **4135.5** for IdaGold II v. a Pounds per Acre over 6/64 value of **3401.21** for IdaGold.

D. The Data Table provided herewith as Attachment B-13 (entitled "2001 PVP~Plant Variety Protection Trial, Burley ID"). This Table sets forth a Pounds per Acre over 6/64 value of **7140.28** for IdaGold II v. a Pounds per Acre over 6/64 value of **6603.98** for IdaGold.

[vi] **IdaGold II has a substantially higher % Screen over 6/64 value compared with IdaGold.**

This key difference is clearly illustrated in:

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A. The Data Table provided herewith as Attachment B-11 (entitled "1996 NCVPT~Northern Colorado Variety Performance Trial, Berthoud CO"). This Table sets forth a % Screen over 6/64 value of **86.25** for IdaGold II v. a % Screen over 6/64 value of **56.75** for IdaGold.

B. The Data Table provided herewith as Attachment B-12 (entitled "1996 SCVPT~Southern Colorado Variety Performance Trial, Center CO"). This table sets forth a % Screen over 6/64 value of **47** for IdaGold II v. a % Screen over 6/64 value of **44.75** for IdaGold.

C. The Data Table provided herewith as Attachment B-10 (entitled "1996 SIVPT~Southern Idaho Variety Performance Trial, Burley ID"). This table sets forth a % Screen over 6/64 value of **94.75** for IdaGold II v. a % Screen over 6/64 value of **93** for IdaGold.

D. The Data Table provided herewith as Attachment B-13 (entitled "2001 PVP~Plant Variety Protection Trial, Burley ID"). This Table sets forth a % Screen over 6/64 value of **94.6667** for IdaGold II v. a % Screen over 6/64 value of **93.3333** for IdaGold.

[vii] **IdaGold II has a significantly higher Test Weight value compared with IdaGold.**

This key difference is clearly illustrated in:

A. The Data Table provided herewith as Attachment B-11 (entitled "1996 NCVPT~Northern Colorado Variety Performance Trial, Berthoud CO"). This Table sets forth a Test Weight value of **50.725** for IdaGold II v. a Test Weight value of **47.5** for IdaGold.

B. The Data Table provided herewith as Attachment B-12 (entitled "1996 SCVPT~Southern Colorado Variety Performance Trial, Center CO"). This table sets forth a Test Weight value of **48.075** for IdaGold II v. a Test Weight value of **47.1** for IdaGold.

C. The Data Table provided herewith as Attachment B-13 (entitled "2001 PVP~Plant Variety Protection Trial,

Burley ID"). This Table sets forth a Test Weight value of **53.4333** for IdaGold II v. a Test Weight value of **52.4667** for IdaGold.

D. The Data Table provided herewith as Attachment B-10 (entitled "1996 SIVPT~Southern Idaho Variety Performance Trial, Burley ID"). This table sets forth a Test Weight value of **52.95** for IdaGold II v. a Test Weight value of **52.475** for IdaGold.

Conclusion: IdaGold II is entirely distinct compared with IdaGold based on all of the information presented above which is clear and convincing. This is of considerable importance in the current case since IdaGold is one of the two closest varieties to IdaGold II as previously stated.

2. **IdaGold II v. C19:**

As noted above in the first portion of this Exhibit which pertains to comparative genetic data, both IdaGold II and C19 are characterized by distinctively different genetic profiles. This data constitutes definitive evidence of the unique nature of IdaGold II relative to C19 and is sufficient, by itself, to support the allowability of IdaGold II as a totally distinct variety compared with C19. However, as supplemental/extra evidence, the following agronomic differences and data are provided concerning IdaGold II v. C19:

[i] **IdaGold II has a significantly shorter Plant Height compared with C19.**

This key difference is clearly illustrated in:

A. The Data Table provided herewith as Attachment B-12 (entitled "1996 SCVPT~Southern Colorado Variety Performance Trial, Center CO"). This Table sets forth a

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Plant Height value of **26** (in.) for IdaGold II v. a Plant Height value of **31.5** (in.) for C19.

B. The Data Table provided herewith as Attachment B-10 (entitled "1996 SIVPT~Southern Idaho Variety Performance Trial, Burley ID"). This Table sets forth a Plant Height value of **27.75** (in.) for IdaGold II v. a Plant Height value of **31** (in.) for C19.

C. The Data Table provided herewith as Attachment B-11 (entitled "1996 NCVPT~Northern Colorado Variety Performance Trial, Berthoud CO"). This Table sets forth a Plant Height value of **22.75** (in.) for IdaGold II v. a Plant Height value of **26.25** (in.) for C19.

[ii] **IdaGold II has a substantially different Color compared with C19.**

This key difference is clearly illustrated in:

A. The Data Table provided herewith as Attachment B-11 (entitled "1996 NCVPT~Northern Colorado Variety Performance Trial, Berthoud CO"). This Table sets forth a Color value of **41** for IdaGold II v. a Color value of **34.75** for C19.

B. The Data Table provided herewith as Attachment B-12 (entitled "1996 SCVPT~Southern Colorado Variety Performance Trial, Center CO"). This table sets forth a Color value of **69.25** for IdaGold II v. a Color value of **63.5** for C19.

C. The Data Table provided herewith as Attachment B-10 (entitled "1996 SIVPT~Southern Idaho Variety Performance Trial, Burley ID"). This table sets forth a Color value of **58.25** for IdaGold II v. a Color value of **57.25** for C19.

[iii] **IdaGold II has a significantly higher Moisture value compared with C19.**

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This key difference is clearly illustrated in:

A. The Data Table provided herewith as Attachment B-11 (entitled "1996 NCVPT~Northern Colorado Variety Performance Trial, Berthoud CO"). This Table sets forth a Moisture value of **10.4** for IdaGold II v. a Moisture value of **9.925** for C19.

B. The Data Table provided herewith as Attachment B-12 (entitled "1996 SCVPT~Southern Colorado Variety Performance Trial, Center CO"). This Table sets forth a Moisture value of **10.975** for IdaGold II v. a Moisture value of **10.675** for C19.

C. The Data Table provided herewith as Attachment B-10 (entitled "1996 SIVPT~Southern Idaho Variety Performance Trial, Burley ID"). This Table sets forth a Moisture value of **10.425** for IdaGold II v. a Moisture value of **10.325** for C19.

Conclusion: IdaGold II is entirely distinct compared with C19 based on all of the information presented above (both agronomic and genetic data) which is clear and convincing.

3. IdaGold II v. Andrea

As noted above in the first portion of this Exhibit which pertains to comparative genetic data, both IdaGold II and Andrea are characterized by distinctively different genetic profiles. This data constitutes definitive evidence of the unique nature of IdaGold II relative to Andrea and is sufficient, by itself, to support the allowability of IdaGold II as a totally distinct variety compared with Andrea. However, as supplemental/extra evidence, the following agronomic differences and data are provided concerning IdaGold II v. Andrea:

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[i] **IdaGold II has a significantly lower Test Weight compared with Andrea.**

This key difference is clearly illustrated in:

A. The Data Table provided herewith as Attachment B-13 (entitled "2001 PVP~Plant Variety Protection Trial, Burley ID"). This Table sets forth a Test Weight value of **53.4333** for IdaGold II v. a Test Weight value of **55.0333** for Andrea.

[ii] **IdaGold II has a significantly different Color compared with Andrea.**

This key difference is clearly illustrated in:

A. The Data Table provided herewith as Attachment B-13 (entitled "2001 PVP~Plant Variety Protection Trial, Burley ID"). This Table sets forth a Color value of **56.6667** for IdaGold II v. a Color value of **64.3333** for Andrea.

[iii] **IdaGold II has a significantly shorter Plant Height compared with Andrea.**

This key difference is clearly illustrated in:

A. The Data Table provided herewith as Attachment B-13 (entitled "2001 PVP~Plant Variety Protection Trial, Burley ID"). This Table sets forth a Plant Height value of **18** (in.) for IdaGold II v. a Plant Height value of **23.3333** (in.) for Andrea.

[iv] **IdaGold II has a substantially higher Plot Yield compared with Andrea.**

This key difference is clearly illustrated in:

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A. The Data Table provided herewith as Attachment B-13 (entitled "2001 PVP~Plant Variety Protection Trial, Burley ID"). This Table sets forth a Plot Yield value of **9.13333** for IdaGold II v. a Plot Yield value of **8.49333** for Andrea.

[v] **IdaGold II yields substantially greater Bushels/Acre (Bu/Ac) compared with Andrea.**

This key difference is clearly illustrated in:

A. The Data Table provided herewith as Attachment B-13 (entitled "2001 PVP~Plant Variety Protection Trial, Burley ID"). This Table sets forth a Bu/Ac value of **150.761** for IdaGold II v. a Bu/Ac value of **140.467** for Andrea.

[vi] **IdaGold II has a substantially higher Pounds per Acre at 12% Water value compared with Andrea.**

This key difference is clearly illustrated in:

A. The Data Table provided herewith as Attachment B-13 (entitled "2001 PVP~Plant Variety Protection Trial, Burley ID"). This Table sets forth a Pounds per Acre at 12% Water value of **7538.07** for IdaGold II v. a Pounds per Acre at 12% Water value of **7023.36** for Andrea.

[vii] **IdaGold II has a substantially higher Pounds per Acre over 6/64 value compared with Andrea.**

This key difference is clearly illustrated in:

A. The Data Table provided herewith as Attachment B-13 (entitled "2001 PVP~Plant Variety Protection Trial, Burley ID"). This Table sets forth a Pounds per Acre over

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6/64 value of **7140.28** for IdaGold II v. a Pounds per Acre over 6/64 value of **6320.47** for Andrea.

[viii] **IdaGold II has a substantially higher % Screen over 6/64 value compared with Andrea.**

This key difference is clearly illustrated in:

A. The Data Table provided herewith as Attachment B-13 (entitled "2001 PVP~Plant Variety Protection Trial, Burley ID"). This Table sets forth a % Screen over 6/64 value of **94.6667** for IdaGold II v. a % Screen over 6/64 value of **90** for Andrea.

[ix] **IdaGold II has a substantially lower Lodging value compared with Andrea.**

This key difference is clearly illustrated in:

A. The Data Table provided herewith as Attachment B-13 (entitled "2001 PVP~Plant Variety Protection Trial, Burley ID"). This Table sets forth a Lodging value of **0** for IdaGold II v. a Lodging value of **20** for Andrea.

Conclusion: IdaGold II is entirely distinct compared with Andrea based on all of the information presented above (both agronomic and genetic data) which is clear and convincing.

4. IdaGold II v. C13

As noted above in the first portion of this Exhibit which pertains to comparative genetic data, both IdaGold II and C13 are characterized by distinctively different genetic profiles. This data constitutes definitive

evidence of the unique nature of IdaGold II relative to C13 and is sufficient, by itself, to support the allowability of IdaGold II as a totally distinct variety compared with C13. However, as supplemental/extra evidence, the following agronomic differences and data are provided concerning IdaGold II v. C13:

[i] **IdaGold II has a significantly higher Pounds per Acre at 12% Water value compared with C13.**

This key difference is clearly illustrated in:

A. The Data Table provided herewith as Attachment B-13 (entitled "2001 PVP~Plant Variety Protection Trial, Burley ID"). This Table sets forth a Pounds per Acre at 12% Water value of **7538.07** for IdaGold II v. a Pounds per Acre at 12% Water value of **6848.56** for C13.

[ii] **IdaGold II has a substantially higher Plot Yield compared with C13.**

This key difference is clearly illustrated in:

A. The Data Table provided herewith as Attachment B-13 (entitled "2001 PVP~Plant Variety Protection Trial, Burley ID"). This Table sets forth a Plot Yield value of **9.13333** for IdaGold II v. a Plot Yield value of **8.32667** for C13.

[iii] **IdaGold II yields substantially greater Bushels/Acre (Bu/Ac) compared with C13.**

This key difference is clearly illustrated in:

A. The Data Table provided herewith as Attachment B-13 (entitled "2001 PVP~Plant Variety Protection Trial, Burley ID"). This Table sets forth a Bu/Ac value of **150.761** for IdaGold II v. a Bu/Ac value of **136.971** for C13.

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[iv] **IdaGold II has a substantially higher Pounds per Acre over 6/64 value compared with C13.**

This key difference is clearly illustrated in:

A. The Data Table provided herewith as Attachment B-13 (entitled "2001 PVP~Plant Variety Protection Trial, Burley ID"). This Table sets forth a Pounds per Acre over 6/64 value of **7140.28** for IdaGold II v. a Pounds per Acre over 6/64 value of **6552.97** for C13.

Conclusion: IdaGold II is entirely distinct compared with C13 based on all of the information presented above (both agronomic and genetic data) which is clear and convincing.

5. IdaGold II v. Crystal (one of the two varieties closest to IdaGold as previously stated.)

As noted above in the first portion of this Exhibit which pertains to comparative genetic data, both IdaGold II and Crystal are characterized by distinctively different genetic profiles. This data constitutes definitive evidence of the unique nature of IdaGold II relative to Crystal and is sufficient, by itself, to support the allowability of IdaGold II as a totally distinct variety compared with Crystal. However, as supplemental/extra evidence, the following agronomic differences and data are provided concerning IdaGold II and Crystal:

[i] **IdaGold II has a significantly different Color compared with Crystal.**

This key difference is clearly illustrated in:

A. The Data Table provided herewith as Attachment B-13 (entitled "2001 PVP~Plant Variety Protection Trial,

Burley ID"). This Table sets forth a Color value of **56.6667** for IdaGold II v. a Color value of **69.6667** for Crystal.

[ii] **IdaGold II has a significantly shorter Plant Height compared with Crystal.**

This key difference is clearly illustrated in:

A. The Data Table provided herewith as Attachment B-13 (entitled "2001 PVP~Plant Variety Protection Trial, Burley ID"). This Table sets forth a Plant Height value of **18** (in.) for IdaGold II v. a Plant Height value of **26** (in.) for Crystal.

[iii] **IdaGold II has a substantially lower Pounds per Acre over 6/64 value compared with Crystal.**

This key difference is clearly illustrated in:

A. The Data Table provided herewith as Attachment B-13 (entitled "2001 PVP~Plant Variety Protection Trial, Burley ID"). This Table sets forth a Pounds per Acre over 6/64 value of **7140.28** for IdaGold II v. a Pounds per Acre over 6/64 value of **7262.64** for Crystal.

[iv] **IdaGold II has a substantially lower % Screen over 6/64 value compared with Crystal.**

This key difference is clearly illustrated in:

A. The Data Table provided herewith as Attachment B-13 (entitled "2001 PVP~Plant Variety Protection Trial, Burley ID"). This Table sets forth a % Screen over 6/64 value of **94.6667** for IdaGold II v. a % Screen over 6/64 value of **96.6667** for Crystal.

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Conclusion: IdaGold II is entirely distinct compared with Crystal based on all of the information presented above which is clear and convincing. This is of considerable importance in the current case since Crystal is one of the two closest varieties to IdaGold II as previously stated.

6. IdaGold II v. Grit

As noted above in the first portion of this Exhibit which pertains to comparative genetic data, both IdaGold II and Grit are characterized by distinctively different genetic profiles. This data constitutes definitive evidence of the unique nature of IdaGold II relative to Grit and is sufficient, by itself, to support the allowability of IdaGold II as a totally distinct variety compared with Grit. However, as supplemental/extra evidence, the following agronomic differences and data are provided concerning IdaGold II and Grit:

[i] **IdaGold II has a significantly different Color compared with Grit.**

This key difference is clearly illustrated in:

A. The Data Table provided herewith as Attachment B-13 (entitled "2001 PVP~Plant Variety Protection Trial, Burley ID"). This Table sets forth a Color value of **56.6667** for IdaGold II v. a Color value of **62.6667** for Grit.

[ii] **IdaGold II has a substantially higher Plot Yield compared with Grit.**

This key difference is clearly illustrated in:

A. The Data Table provided herewith as Attachment B-13 (entitled "2001 PVP~Plant Variety Protection Trial, Burley ID"). This Table sets forth a Plot Yield value of

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9.13333 for IdaGold II v. a Plot Yield value of **8.72667** for Grit.

[iii] **IdaGold II has a substantially higher Test Weight compared with Grit.**

This key difference is clearly illustrated in:

A. The Data Table provided herewith as Attachment B-13 (entitled "2001 PVP~Plant Variety Protection Trial, Burley ID"). This Table sets forth a Test Weight value of **53.4333** for IdaGold II v. a Test Weight value of **51.8667** for Grit.

[iv] **IdaGold II yields substantially greater Bushels/Acre (Bu/Ac) compared with Grit.**

This key difference is clearly illustrated in:

A. The Data Table provided herewith as Attachment B-13 (entitled "2001 PVP~Plant Variety Protection Trial, Burley ID"). This Table sets forth a Bu/Ac value of **150.761** for IdaGold II v. a Bu/Ac value of **144.393** for Grit.

[v] **IdaGold II has a substantially higher Pounds per Acre at 12% Water value compared with Grit.**

This key difference is clearly illustrated in:

A. The Data Table provided herewith as Attachment B-13 (entitled "2001 PVP~Plant Variety Protection Trial, Burley ID"). This Table sets forth a Pounds per Acre at 12% Water value of **7538.07** for IdaGold II v. a Pounds per Acre at 12% Water value of **7219.65** for Grit.

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[vi] **IdaGold II has a substantially higher Pounds per Acre over 6/64 value compared with Grit.**

This key difference is clearly illustrated in:

A. The Data Table provided herewith as Attachment B-13 (entitled "2001 PVP~Plant Variety Protection Trial, Burley ID"). This Table sets forth a Pounds per Acre over 6/64 value of **7140.28** for IdaGold II v. a Pounds per Acre over 6/64 value of **6200.19** for Grit.

[vii] **IdaGold II has a substantially higher % Screen over 6/64 value compared with Grit.**

This key difference is clearly illustrated in:

A. The Data Table provided herewith as Attachment B-13 (entitled "2001 PVP~Plant Variety Protection Trial, Burley ID"). This Table sets forth a % Screen over 6/64 value of **94.6667** for IdaGold II v. a % Screen over 6/64 value of **85.6667** for Grit.

[viii] **IdaGold II has a substantially shorter Plant Height compared with Grit.**

This key difference is clearly illustrated in:

A. The Data Table provided herewith as Attachment B-13 (entitled "2001 PVP~Plant Variety Protection Trial, Burley ID"). This Table sets forth a Plant Height value of **18** (in.) for IdaGold II v. a Plant Height value of **20** (in.) for Grit.

[ix] **IdaGold II has a substantially lower Lodging value compared with Grit.**

This key difference is clearly illustrated in:

A. The Data Table provided herewith as Attachment B-13 (entitled "2001 PVP~Plant Variety Protection Trial, Burley ID"). This Table sets forth a Lodging value of **0** for IdaGold II v. a Lodging value of **26.6667** for Grit.

Conclusion: IdaGold II is entirely distinct compared with Grit based on all of the information presented above (both agronomic and genetic data) which is clear and convincing.

Overall Conclusions

It is overwhelmingly clear from the above-listed data that IdaGold II is completely distinguishable from (1) Andrea; (2) C13; (3) Crystal; (4) Grit; (5) C19; and (6) IdaGold (e.g. IdaGold I) in many different ways, thereby confirming the novelty and distinctness of IdaGold II under all statutory guidelines. Accordingly, Applicant/Owner Coors Brewing Company is entitled to Plant Variety Protection on IdaGold II and should any further information be needed, it will be provided immediately upon request.

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Isolation, characterizaton, and inheritance of microsatellite loci in alpine larch and western larch

P.D. Khasa, C.H. Newton, M.H. Rahman, B. Jaquish, and B.P. Dancik

Abstract: Microsatellite loci or simple sequence repeat loci (SSRs) were isolated in alpine larch (*Larix lyallii* Parl.) and western larch (*Larix occidentalis* Nutt.). In total, 14 SSR loci were characterized; two [$(TCT)_{4, A7}$] came from published *Larix* DNA sequence data, one $(CA)_{17}$ was obtained from a partial non-enriched alpine larch total genomic DNA library, and the remaining 11 loci were obtained from larch genomic DNAs enriched for $(CA)_n$ repeats. The SSR regions in these clones could be divided into three categories: perfect repeat sequences without interruption, imperfect repeat sequences with interruption(s), and compound repeat sequences with adjacent tandem simple dinucleotides. Eight of the 14 loci analyzed were found to be polymorphic and useful markers after silver-staining polyacrylamide gel electrophoresis. In addition, several SSR primers developed for alpine larch were able to successfully amplify polymorphic loci in its related species, western larch, and among other closely related taxa within the *Larix* genus. The inheritance of microsatellite loci was verified by analysis of haploid megagametophyte and diploid embryo tissues of progeny obtained from controlled crosses between western larch and alpine larch. All microsatellite loci analyzed had alleles that segregated according to expected Mendelian frequencies. Two species-specific markers (*UAKLly10a* and *UAKLla1*) allow easy and rapid identification of specific genetic entry of alpine larch and western larch at any stage in the sporophyte phase of the life cycle. Therefore, these markers are efficient in identifying the parental species and to validate controlled crosses between these two closely related species. These results are important in tree improvement programs of alpine larch and western larch aimed at producing genetically improved hybrid stock for reforestation in Western Canada and U.S.A.

Key words: database search, enriched library, inheritance, *Larix*, microsatellites, simple sequence repeats, PCR.

Résumé : Des locus microsatellites aussi appelés des répétitions de séquences simples (SSRs) ont été isolés chez le mélèze subalpin (*Larix lyallii* Parl.) et le mélèze de l'Ouest (*Larix occidentalis* Nutt.). Au total, 14 locus SSR ont été caractérisés: deux locus [$(TCT)_{4, A7}$] ont été obtenus des séquences d'ADN publiées du mélèze, un locus $(CA)_{17}$, a été obtenu à partir d'une banque génomique partielle d'ADN total du mélèze subalpin et les 11 autres locus à partir d'une banque génomique d'ADN du mélèze subalapin, enrichie avec des répétitions de séquences simples $(CA)_n$. Les régions SSR de ces clones ont été divisées en trois catégories: les séquences répétées parfaites sans interruption, les séquences répétées imparfaites avec interruption(s), et les séquences composées avec des dinucléotides simples adjacents en tandem. Parmi les 14 locus analysés en utilisant le gel de polyacrylamide coloré à l'argent, huit se sont avérés polymorphes et utiles comme marqueurs génétiques. Par surcroît, plusieurs paires d'amorces synthétisées en vue de l'amplification de microsatellites chez le mélèze subalpin, étaient capables d'amplifier avec succès des locus polymorphes chez son espèce voisine, le mélèze de l'Ouest, et chez plusieurs autres espèces voisines du genre *Larix*. L'hérédité de ces locus microsatellites a été vérifiée par l'analyse des tissus des mégagamétophytes haploïdes et des embryons diploïdes de la progéniture obtenue à partir de croisements contrôlés entre le mélèze subalpin et le mélèze de l'Ouest. Tous les locus microsatellites analysés avaient des allèles ségrégeant selon les fréquences Mendéliennes espérées. Deux marqueurs spécifiques (*UAKLly10a* et *UAKLla1*) de chacune de deux espèces, permettent une identification facile et rapide du matériel génétique du mélèze subalpin et du mélèze de l'Ouest quelque soit le stade de développement de ces deux espèces de mélèze. Donc, ces marqueurs sont aussi efficaces pour identifier les espèces parentales et valider les croisements contrôlés entre ces deux espèces voisines. Ces résultats sont importants dans des programmes d'amélioration génétique du mélèze subalpin et du mélèze de l'Ouest visant la production de matériel hybride

Corresponding Editor: J.B. Bell.

Received June 3, 1999. Accepted November 3, 1999. Published on the NRC Research Press web site on April 28, 2000.

P.D. Khasa,[1] **M.H. Rahman, and B.P. Dancik.** Department of Renewable Resources, University of Alberta, Edmonton, AB T6G 2H1, Canada.
C.H. Newton. B.C. Research Inc., 3650 Wesbrook Mall, Vancouver, BC V6S 2L2, Canada.
B. Jaquish. Research Branch, B.C. Ministry of Forests, Kalamalka Research Station, 3401 Reservoir Road, Vernon, BC V1B 2C7, Canada.

[1]Author to whom all correspondence should be addressed (e-mail: damase.khasa@ualberta.ca).

génétiquement supérieur pour le reboisement dans l'Ouest du Canada et des États-unis.

Mots clés : banque des données, banque génomique enrichie, hérédité, *Larix*, microsatellites, répétitions de séquences simples, PCR.

Introduction

The larch genus, *Larix*, the only deciduous needle-leaf conifer in the family Pinaceae, comprises 10 commonly recognized species (Schmidt 1995), divided in two sections, *pauciseriales* and *multiseriales*. Larch resources essentially encircle the Northern Hemisphere, stretching from eastern Siberia westward across Eurasia (except Scandinavia), resuming in eastern North America and westward to Alaska, where except for the Bering sea, they reach eastern Siberia. These resources play an economically and ecologically important role in the Northern Hemisphere. The importance of larch resources has been emphasized in a comprehensive and international examination of *Larix* species of the world (Schmidt and McDonald 1995). Sustainable use of larch resources require their genetic dissection, which could be achieved through the use of genetic markers.

Since the 1960s, isozyme analysis has been the quickest and least expensive technique being used to assess the genetic diversity, population structure, and mating systems of plant species (Hamrick and Godt 1990). This technique has been used to measure genetic variation of several larch species including western larch (Fins and Seeb 1986; Jaquish and El-Kassaby 1998), tamarack, *Larix laricina* (Du Roi) K. Koch. (Cheliak et al. 1988), and several Eurasian larch species (Semerikov and Matveev 1995; Semerikov and Lascoux 1999; Semerikov et al. 1999). Allozyme markers can detect variation only for protein-coding genes, and only a fraction of all mutational events (i.e., those changing protein mobility in a gel) can be resolved, while DNA-based markers overcome these disadvantages (Strauss et al. 1992; Hillis et al. 1996). Genetic relationships among larch species have also been studied by analyzing restriction fragment length polymorphism (RFLP) in the chloroplast DNA (Qian et al. 1995).

New classes of polymerase chain reaction (PCR)-based molecular markers have been developed over the past few years, including random amplified polymorphic DNA (RAPD, Williams et al. 1990) or arbitrarily primed PCR (Welsh et al. 1991), DNA amplification fingerprinting (Caetano-Anollés et al. 1991), sequence-tagged sites (STSs, Inoue et al. 1994), amplified fragment length polymorphism (AFLP, Vos et al. 1995), and microsatellites (Tautz 1989; Litt and Luty 1989; Webber and May 1989; Hughes and Queller 1993). Microsatellite markers, also called simple sequence repeats (SSRs) or simple tandem repeats (STRs), consist of tandem repeats of relatively short sequences (1–7 bases long) that are highly polymorphic, frequently codominant, and are spread throughout the genomes of virtually all eucaryotic organisms (Tautz 1989; Webber and May 1989). Microsatellite length polymorphism technology has been applied to forensics (Queller et al. 1993; Paetkau and Strobeck 1994), the analysis of the genetic structure, parentage, and gene flow of plant and animal populations (Paetkau and Strobeck 1994; Powell et al. 1995; Slatkin 1995), evolutionary and ecological research (Queller et al. 1993; Jarne and Lagoda 1996), and genome mapping (Love et al. 1990).

Two main advantages of microsatellite loci are that they are generally highly variable codominant loci and that they are PCR-based markers. Attempts to detect losses of variation in isolated patches or to make comparisons of genetic variability between populations would be enhanced through the use of more variable loci. Because of the simplicity of assay, codominance, and strong discriminatory power due to high allelic diversity, microsatellites are becoming a popular tool in molecular genetic analysis (Tautz 1989; Webber and May 1989; Queller et al. 1993). Genetic analysis using microsatellites involves PCR amplification of DNA using oligonucleotide primers complementary to a flanking region of a given microsatellite locus, size fractionation of the amplified product by polyacrylamide gel electrophoresis (PAGE), and detection of DNA fragments on the gel through staining or via automated systems. The relatively simple interpretation and genetic analysis of single-locus markers make them very useful in population genetics studies (Powell et al. 1995), and for map construction and DNA typing (Thomas and Scott 1993). A disadvantage, however, at least in plants, and conifers in particular, has been the considerable time required for development of microsatellite loci.

Most microsatellites are identified from screening small-insert (200–700 bp) genomic libraries with oligonucleotide probes. The number of positive clones identified is a function of the abundance of the target microsatellite repeats in the genome under study. This problem is particularly acute in plants where the number of microsatellite repeats detected is 5 to 10 times less compared to mammals (Lagercrantz et al. 1993). To overcome this problem, various enrichment procedures of genomic libraries have been proposed that can yield clones containing a variety of microsatellites (Edwards et al. 1996). We have used two strategies (database search and cloning) to develop microsatellite markers for members of the only existing deciduous conifer of the genus *Larix* in the family Pinaceae (Schmidt and McDonald 1995). The microsatellite loci from *Larix* were characterized and their inheritance verified using megagametophyte (1*N*) and embryo (2*N*) tissues from controlled cross seeds between alpine larch and western larch. We also tested their utility in amplification of DNA of other larch species. The SSR markers developed are well suited for population genetic studies, evolutionary and ecological research, and genetic improvement and gene management programs of alpine larch, western larch, and related species and for other genomics applications.

Materials and methods

Two strategies (database search and cloning of microsatellite loci) were used to develop microsatellite markers for alpine larch and western larch.

Database search

Database searches (in GenEMBLPlus) for *Larix* spp. were performed using the Genetics Computer Group program (GCG 1994). With the GCG software, we searched for all types of mono- and dinucleotide repeats with a minimum number of seven repeats; and

for all types of tri- and tetranucleotide repeats, with a minimum number of four repeats without mismatch, using the FINDPATTERNS program.

Cloning of microsatellite loci

Plant materials and DNA extraction

DNA was extracted from twigs of alpine larch following a modified CTAB (cetyltrimethylammonium bromide) method (Bousquet et al. 1990). Three grams of twigs were cut into pieces and ground to a powder in liquid nitrogen using a coffee grinder and 15 mL homogenized in a preheated CTAB extraction buffer (37°C) [2% (w/v) CTAB (Sigma), 1.4 M NaCl, 20 mM EDTA, 100 mM Tris, 0.2% β-mercaptoethanol, pH 9.5] in 50-mL Falcon® tubes (Fisher Scientific), and swirled gently to mix. The homogenate was then incubated in a water bath at 60°C for 1 h with occasional gentle swirling. Twenty microlitres of RNase A (10 mg/mL) from bovine pancreas (Sambrook et al. 1989) was added to the samples, and incubated for a further 1 h at 37°C. Following incubation, 15 mL of chloroform : octanol or isoamyl alcohol (24:1, (v/v)) was added, mixed gently but thoroughly, and centrifuged (4000 rpm, 15 min, 15°C). The upper aqueous phase was gently removed with a wide-bore pipette, and transferred to a clean, glass centrifuge tube containing 15 mL of cold isopropanol to precipitate DNA by gentle mixing. If no precipitation was observed, the samples were left overnight at –20°C and DNA recovered by gentle centrifugation at 500 rpm for 2 min. The DNA pellet was hooked out and washed in 1 mL of buffer [76% (v/v) ethanol, 10 mM ammonium acetate] for 20 min or wash buffer was added to the diffuse and loose precipitate and swirled gently to resuspend the DNA. Ammonium acetate buffer was discarded and the DNA pellet was dried and dissolved in approximately 300–600 μL of TE buffer (Sambrook et al. 1989). Using TE buffer (with EDTA, which stabilizes the nucleic acids), the DNA samples can be stored in stable condition at –20°C for at least 4 years (Khasa and Dancik 1996). The concentration of DNA samples was determined by spectrophotometric measurement at 260 nm and by comparison with known amounts of a low DNA mass ladder (Gibco–BRL, Life Technologies, Gaithersburg, Md.) on a 2% agarose gel stained with ethidium bromide. Finally, the DNA concentration was adjusted to the desired concentration for use in PCR amplification by dilution with sterilized double-distilled water.

For cloning purposes, the larch DNA was further purified by cesium chloride density gradient ultracentrifugation (Sambrook et al. 1989). Five hundred microlitres of alpine larch genomic DNA from one single tree of alpine larch was mixed in a centrifuge tube with 4.15 g of CsCl, NET (NaCl/EDTA/Tris) buffer to 4 mL and 100 μL of EtBr (10 mg/μL). The tube was heat-sealed, ultracentrifuged overnight in a Beckman VTi 65.2 rotor class H (Palo Alto, Calif.) at 65 000 rpm, and the DNA band collected. The samples were then extracted with isobutanol to remove EtBr, following which they were suspended in TE buffer and spun at 6000 rpm in a millipore tube (10 000 NMWL filter unit, ultrafree-MC), to remove any remaining CsCl. The DNA was rinsed with TE twice to remove CsCl, then collected from the filter and transferred to a clean tube. The purified DNA was then quantified by using a UV spectrophotometer at 260 nm and 280 nm and by gel electrophoresis against a mass ladder. A genomic library was constructed with this DNA as described below using both non-enriched and enriched libraries.

Genomic DNA library construction and screening

We have used both non-enriched and enriched library strategies. In the non-enriched library strategy, genomic DNA of alpine larch was digested with *Sau*3AI. Size-selected (approximately 300–700 bp) fragments were purified from an agarose gel with the Prep-A-Gene kit (BioRad, Hercules, Calif.) and then ligated into *Bam*HI-digested and dephosphorylated pUC18 (Amersham Pharmacia Biotech, Baie-d'Urfé, Que.). Following transformation of competent *Escherischia coli* DH5α cells, the size-selected library was screened by colony hybridization at 55°C using ^{32}P-labelled poly [$(GA)_{15}$ + $(CA)_{15}$] in 6× SSPE ($NaCl/NaH_2PO_4{\cdot}H_2O$/EDTA), 5× Denhardt's solution, 1% SDS (sodium dodecyl sulfate), with 3 post-hybridization washes in 0.5× SSC (NaCl/sodium citrate), 0.1% SDS (Sambrook et al. 1989). Putative positive colonies were replated and a second round of screening was carried out to confirm their positive status by PCR amplification of the inserts from forward and reverse primers flanking the cloning site.

For the enriched library, microsatellite markers were isolated from genomic DNA using modifications of published biotin-enrichment strategies (Kijas et al. 1994; Edwards et al. 1996). Approximately 0.5–1.0 μg of total larch genomic DNA was incubated overnight at 37°C in a 60 μL reaction mixture containing 10 mM Tris–acetate (pH 7.5), 10 mM magnesium acetate, 50 mM potassium acetate, 0.5 mM adensoine triphosphate (rATP), 5 mM dithiothreitol, 20 μg/mL bovine serum albumin (BSA), 30 U *Hae*III (Pharmacia), 10 U *Psb*AI (New England Biolabs), 600 U T4 DNA ligase (New England Biolabs), and 60 pmol of an equimolar mixture of the oligonucleotides M28, 5′-CTCTTGCTTGAATTCGGACTA and M29, 5′-pTAGTCCGAATTCAAGCAAGAGCACA. A portion (5–10 μL) of the above digestion–ligation reaction mixture was denatured (5 min at 95°C) with 0.5 to 1 pmol of 5′ biotin-labeled oligonucleotide $(TG)_{12}$ and hybridized at 55°C for 15 min. The hybridization was terminated by quick-chilling on ice water and either used immediately or stored at –20°C. Biotin-captured larch DNA was then selected using Dynal M280 streptavidin magnetic beads (Promega, Madison, Wis.) according to the manufacturer's instructions, washed twice in 0.1× TEN (Tris/EDTA/NaCl) solution and a portion (1/50) amplified using the oligonucleotide M30 (5′-CTCTTGCTTGAATTCGGACTACC). For cloning into plasmid vectors, the amplification mixture was purified using silica-based retention columns (FMC or Qiagen) and then digested with *Eco*RI. The digestion reaction was purified as before and a portion was then ligated into *Eco*RI-digested pGEM3Z+ (Promega). Ligated DNAs were transformed into *E. coli* (strain SURE™, Stratagene, La Jolla Calif.) and screened by colony hybridization with ^{32}P-labelled $(AC)_{12}$. Approximately 10–20% of the cells contained inserts that hybridized with the probe. Positive colonies were picked and sequencing templates prepared by amplification using the forward and reverse universal M13 primers.

Primer design and PCR protocol

Sequencing of positive clones from both non-enriched and enriched libraries was performed using an ABI 373 automated DNA sequencer (PE Biosystems, Foster City, Calif.). The primers used for sequencing were F (–21m13) and R (m13R). Specific sets of primers (18–20 bp), complementary to the flanking microsatellite regions obtained from database search and cloning techniques, were designed with the aid of the program PRIME of the GCG sequence analysis software (GCG 1994). To test their heterologous nature, these primers were assayed in a PCR on several larch species (*L. lyallii*, *L. occidentalis*, *L. eurolepsis*, *L. laricina*, *L. leptolepis*, *L. mastersiana*, *L. gmelinii*, and *L. olgensis*) using the same PCR protocol. PCRs were carried out in a 10-μL volume containing 20 ng of genomic DNA, 200 μM of each dNTP, 2.5 pmol of each primer, PCR buffer (50 mM KCl + 10 mM Tris–HCl, pH 8.3), 2 μg BSA, 1.5 mM $MgCl_2$, and 1 U of Amplitaq® DNA polymerase. A negative control was used in every experiment to test for the presence of DNA contamination of reagents and reaction mixtures. In general, the PCR protocol of microsatellite analysis was optimized on a GeneAmp 9600® thermal cycler (Perkin–Elmer Cetus, Norwalk, Conn.) with a touchdown program as follows: 3 min pre-incubation at 94°C followed by 2 cycles of 30 s

each at 94°C (denaturation), 60°C or 66°C (annealing), and 72°C (extension); 11 cycles of 15 s each at 94°C, 60°C, or 66°C, 72°C with stepwise lowering of the annealing temperature from 60°C to 55°C or 66°C to 61°C; and 27 cycles of 15 s each at 94°C, 54°C or 60°C, and 72°C, followed by incubation at 72°C for 3 min as a final extension step. The PCR products were stored at –20°C until use for gel electrophoresis. The PCR products along with 100-bp ladder-DNA sizing markers (Promega), were electrophoresed in 2% agarose gel in 1× TBE (Tris–borate/EDTA) buffer into which the ethidium bromide dye (0.5 μg/mL) was incorporated. The PCR products that amplified cleanly at or near the expected size were tested for their polymorphism on 6% denaturing polyacrylamide gels in 8 M urea and 1× TBE buffer run at 55 W constant power for 3 h. PCR products were denatured by adding 1 volume (10 μL) of fresh SSRP loading dye (10 mM NaOH, 95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol) to 1 volume of PCR sample in a microtiter plate, mixed well, heated to 95°C for 5 min, and placed on ice. Gels were fixed and stained with silver nitrate using a DNA Silver Staining System kit (Promega Silver Sequence™ Staining protocol) as modified by Echt et al. (1996). Sizes were estimated with 100 and 20-bp ladder-DNA sizing markers (Gensura, Bio/Can Scientific) and a 1-bp ladder obtained from an M13 mp18 sequencing reaction.

Inheritance of microsatellite loci

Hybrid seeds were obtained by cross-pollination in 1995 at Carlton Ridge alpine larch stand in Montana, U.S.A., between female alpine larch parent trees and a polymix of western larch male parents. Seeds were surface-sterilized with 2% sodium hypochlorite for 10 min, washed 6 times with sterile distilled water and germinated on wet filter paper in Petri dishes. When seeds had just started to germinate (at approximately 2.5 weeks), the seed coat was removed and embryo and megagametophyte were separated. DNA of mother trees was extracted from needles as above. Each embryo or megagametophyte was transferred in a 1.5-mL sterile microcentrifuge tube containing 30 μL of wash buffer (50 mM Tris–Cl pH 8.0, 25 mM EDTA, 0.35% sorbitol, 0.1% 2-mercaptoethanol) and incubated overnight at room temperature before homogenization with a sterile, disposable, plastic pestle. Two-hundred thirty microlitres of wash buffer and 50 μL of 5% sarcosyl were added to the homogenate, well mixed, and incubated at room temperature for 5 min. Forty microlitres of 5M NaCl, and 30 μL of 8.6% CTAB in 0.7 mM NaCl were then added, well mixed and incubated at 65°C for 15 min. Following incubation, 350 μL of phenol : chloroform : isoamyl alcohol (25:24:1, (v/v/v)) was added, mixed gently but thoroughly, and centrifuged at room temperature (14 000 rpm, 10 min). The top aqueous layer (250–300 μL) was removed and extracted with 1 volume of chloroform : isoamyl alcohol followed by centrifugation as above for 10 min. The top layer (about 250 μL) was again removed and 2 volumes of ice-cold 95% ethanol were added followed by an incubation at –80°C for 20 min, or overnight at –20°C to precipitate the DNA. The samples were spun down for 10–15 min as above and the alcohol discarded. Three hundred microlitres of ice-cold 95% ethanol were added, and the samples incubated at –80°C for 20 min, spun down for 10–15 min as above, and the alcohol discarded. The DNA pellet was dried and dissolved in approximately 30 μL of 1× TE buffer.

To identify heterozygous parent trees for each SSR locus, 8 megagametophytes and 8 embryos were genotyped from each of 14 collected trees (#11–#24).

An expected $(½)^{8-1}$ or 0.78% of the heterozygotes would be misclassified in this way, assuming 1:1 segregation at heterozygous loci. For this study, 6 heterozygous trees were selected and at least 30 haploid megagametophytes from each tree were analyzed. The *G* statistic, which has an approximate chi-square distribution with 1 d.f. (Sokal and Rohlf 1981) was used to detect deviation from the expected 1:1 segregation ratio. We performed the G_H test to determine heterogeneity among families. Total *G* (G_T) was also computed by adding the *G* for heterogeneity (G_H) and the pooled *G* (G_P). Because the actual type I error of *G* tests tends to be higher than the intended level, *G* was calculated applying Williams' correction as $G_{adj} = G/q$, to obtain a better approximation to the chi-square distribution (Sokal and Rohlf 1981). Williams' correction was computed as $q = 1 + (a^2 - 1)/6n(a - 1)$, where *a* is the number of classes and *n* represents the total sample size (Sokal and Rohlf 1981). The effect of this correction is to reduce the observed value of *G* slightly.

Results

Isolation and characterization

In total, 14 microsatellite regions were isolated and characterized. Two out 14 microsatellite loci (14.29%) were obtained from data search while one (7.14%) and 11 (78.57%) were derived from non-enriched and enriched genomic libraries, respectively. For larch, indeed, very little DNA sequence information is available in public molecular databases. The search in the GenEMBLPlus database for larch (Table 1) revealed one trinucleotide repeat with a minimum number of four repeats and one short mononucleotide tract with a minimum number of seven repeats from the sequences of the *rbcs* gene (*UAKLla1* and *UAKLla2* loci). The 12 additional microsatellites in Table 1 were obtained from cloned restriction fragments of *Larix* genomic DNA. Only one microsatellite genomic clone was obtained from *Sau*3AI colonies after screening with $[(CA)_{15} + (GA)_{15}]$ probes. The remaining 11 were derived from plasmid *Hae*II libraries enriched for $(CA)_{12}$ repeats. Screening of the partial non-enriched genomic library did not yield many positive clones and microsatellites, with only one positive clone being found employing this strategy. The enriched library yielded more clones that contained a variety of microsatellites as compared to the non-enriched library. Sequencing of at least 10 clones, chosen at random, showed that the insert size ranged from 300 to 700 bp. As expected, most of the identified repeat units found were dinucleotide repeats (4–20 repeat units). The simple sequence repeats found were divided into three categories (Table 1): six perfect repeat sequences without interruption and without adjacent repeats of another sequence [e.g. $UAKLly_7$, $(TG)_8$], four compound (perfect and (or) imperfect) repeat sequences with adjacent tandem simple repeats of a different sequence [e.g., $UAKLly_3$, $UAKLly_{13}$], and four imperfect repeat sequences, with one or more interruptions in the run of repeats [e.g., $UAKLly_{14}$].

Most of the primer pairs tested under optimal conditions for alpine larch and western larch gave one single band at the expected size of the product on horizontal agarose gel that is indicative of single microsatellite locus (Table 1). Very few primer pairs used produced polymorphic bands in the different larch species tested. Eight out of the 14 loci analyzed using silver-staining polyacrylamide gel were found sufficiently polymorphic and hence informative in population genetics studies (Tables 1 and 2). The levels of polymorphism were not determined for two SSR loci (*UAKLly3* and *UAKLly8*) with an expected size product of 500 and 401 bp, respectively, since it is difficult to resolve such large fragments by PAGE. Primer pairs assayed on several larch species produced a PCR product in at least six related larch

Table 1. Attributes of the larch simple sequence repeats.

Repeats and category[a]	Primer sequences	GenBank Acc. No., Library clone	Locus	Sugg. annealing temp. (°C)	Annealing temp. (°C)[b]	Exp. prod. length (bp)[c]	Remarks
$(TCT)_{4,\ perfect}$	(F) ATCTCCTTCATCGTCCAC (R) CCCCAACTAATACCTAATCTAC	X54464, *L. laricina*	*UAKLLA1*	53	60 ↓ 54	181	Monomorphic
$(A)_{7,\ perfect}$	(F) CCCACTGCTCAAATGGAAG (R) TTTGGGGTCATTGTCTGC	X16039, *L. laricina*	*UAKLla2*	52	60 ↓ 54	77	Monomorphic
$(CA)_{17,\ perfect}$	(F) CGTAGTCACCAATTAAAGTCTA (R) CGTTAGTATGATTTGTATGTTTAT	LLY1 (NEL)	*UAKLLY1*	47	60 ↓ 54	100	Monomorphic
$(CA)_{5\ GA}$ $(CA)_{4,\ imperfect}$	(F) CGAAAGCGAAGAGAGTATCG (R) GTTCCCAAGGAGAAACCCTA	LLY2 (EL)	*UAKLly2*	55	60 ↓ 54	260	Polymorphic
$(GT)_{17,\ perfect}$	(F) AGTTGTACTGTGTGTTGGTC (R) CTGCCTCAACCACTTCTTC	LLY6 (EL)	*UAKLLY6*	48	60 ↓ 54	247	Polymorphic
$(TG)_{8,\ perfect}$	(F) GATTACATCGTGGGTAGGAC (R) AAGTGATTTGGTGTGGTGAC	LLY7 (EL)	*UAKLly7*	50	60 ↓ 54	186	Polymorphic
$(T)_8\ldots(TG)_{9,\ compound\ imperfect}$	(F) GGGGATTTTAGAGCAATAC (R) CCAACAAACAACATCACAC	LLY9 (EL)	*UAKLly9*	54	60 ↓ 54	219	Polymorphic
$(CA)_5AA(CA)_{7,\ imperfect}$	(F) TGGTCGGATTGAGTGAAG (R) ACCCATCCCATGATAGGAG	LLY10 (EL)	*UAKLly10a*	54	50	281	Polymorphic
$(CA)_{10,\ perfect}$	(F) TGGGATGGGTGAAATAGAAG (R) TTCAATCCTCCTCTGGGTC	LLY10 (EL)	*UAKLly10b*	60	66 ↓ 60	119	Polymorphic
$(AT)_5(GT)_{20}(GA)_6(A)_{7,\ compound\ imperfect}$	(F) TCTGTTTACCATCCATAAATC (R) CCACAACCCATTTTAATATC	LLY13 (EL)	*UAKLly13*	60	66 ↓ 60	166	Polymorphic
$(CA)_{10}TA(CA)_4TA(CA)_5$, imperfect	(F) CCAAGCTCCACACATTAAC (R) ATTATTAAGTGGAAGAGGCTC	LLY14 (EL)	*UAKLly14*	60	66 ↓ 60	254	Polymorphic
$(TC)_6(AC)_8NNN(AC)_{12,\ compound\ (perfect)+\ imperfect}$	(F) CCAACCACGGAGAGGGTGAG (R) CACATGATCCCCTTGTACGA	LLY4 (EL)	*UAKLly4*	65	66 ↓ 60	250	Multi-locus
$(TA)_9(TG)_{16,\ compound\ (perfect)}$	(F) GTCCAAGGAAATCCCCAATC (R) CCACCGATGGTCCCTATAAC	LLY3 (EL)	*UAKLly3*	55	ND	500	ND
$(CA)_5AA(CA)_{7,\ imperfect}$	(F) AATTTTGACTACCATGAATTAG (R) GTGTATGGGATCATCCTC	LLY8 (EL)	*UAKLly8*	60	ND	401	ND

Note: NEL, non-enriched library; EL, enriched library. The nomenclature of microsatellite loci follows this convention: the name of institute or location of origin and author, and the species, followed by a numerical designator. ND, not determined.

[a]Repeat sequence categories are according to Weber (1990).

[b]Touch-down temperatures (see also Materials and methods).

[c]Expected PCR product length.

Table 2. Amplification of allele variants (in base pairs) of *Larix* spp. using different larch SSR primers.

Locus Name	*L. lyallii*	*L. occidentalis*	*L. eurolepsis*	*L. laricina*	*L. leptolepis*	*L. mastersiana*	*L. gmelinii*	*L. olgensis*	Product size range (bp)
UAKLla1	175^{++}	178^{++}	175^{++}	175^{++}	175^{++}	175^{+}	175^{+}	175^{++}	175–178
UAKLly2	259^{++}	274^{++}	261^{++}	270^{++}	276^{+}	250^{++}	260^{++}	272^{+}	250–276
		259^{++}	256^{++}						
UAKLly6	233^{++}	227^{++}	264^{++}	237^{++}	256^{++}	214^{+}	231^{++}	236^{++}	214–264
	225^{++}		235^{++}	230^{+}			223^{++}	226^{++}	
UAKLly7	182^{++}	186^{++}	188^{++}	186^{++}	188^{+}	186^{+}	190^{++}	188^{++}	182–190
			186^{++}		186^{+}		186^{++}	186^{++}	
UAKLly10a	274^{++}	298^{++}	330^{++}	292^{++}	330^{++}	298^{++}	280^{++}	298^{+}	274–330
		294^{++}	274^{++}	274^{++}	326^{++}				
UAKLly13	162^{++}	182^{++}	158^{++}	186^{+}	159^{++}	180^{+}	154^{+}	159^{++}	154–186
				172^{+}					
UAKLly14	252^{++}	218^{++}	266^{+}	272^{++}	–	–	286^{++}	264^{+}	216–286
	216^{++}	208^{++}	242^{+}					224^{+}	
UAKLly10b	119^{++}	117^{++}	118^{++}	118^{+}	–	–	119^{+}	–	115–119
			116^{+}				115^{++}		
UAKLly9	216^{++}	220^{++}	206^{++}	216^{++}	–	–	–	204^{++}	192–217
	202^{++}	202^{++}		202^{++}					
UAKLly4	ML	ML	ML	ML	–	–	ML	ML	ML

Note: ++, excellent amplification; +, good amplification; –, no amplification; ML, multilocus. Since the amplification was based on only one individual, the presence of two alleles confirms polymorphism of the locus while one allele doesn't necessarily infer monomorphic locus.

species, though the extent of this success varied (Table 2). Using the same PCR protocol, some of the primers showed positive amplification with all eight larch species tested, while other primers did not amplify DNA from all larch species. Therefore, these primers could possibly be optimized for use in other related larch species. In some cases, more intense bands were associated with faint bands, possibly resulting from polymerase slippage (Tautz 1989) during PCR, especially for dinucleotide repeats. Trinucleotide repeat SSR markers (e.g., *UAKLla1* locus, Fig. 2A and 2B), however, were easy to score because of a lower incidence of stutter bands and larger unit allelic size differences. Even though this study is not intended to be a population survey, it was clear that the levels of polymorphism (e.g., allele numbers) detected in western larch were higher than in alpine larch. Obviously, with only one individual analyzed for the other larch species (Table 2), it was not possible to infer the levels of polymorphism.

Segregation of polymorphic microsatellite loci

Segregation of polymorphic alleles was checked by analyzing amplification products of DNA templates from single megagametophytes of control-pollinated full-sib arrays from alpine larch and western larch from heterozygous mother trees (Table 3; Fig. 1A and 1B). Observed segregation ratios for six SSR loci examined were, in all cases, in agreement with the expected 1:1 segregation ratio of simple codominant Mendelian inheritance (Table 3). Although segregation data were not available for monomorphic loci, and for low polymorphism loci, the observation that the SSR phenotypes of embryos were consistent with a single locus interpretation and (or) that their inheritance was documented in either conifers made it possible. SSR loci with putative null alleles were not included in this study because of potential distortion from the Mendelian expectations (Gullberg et al. 1997). Two SSRs (*UAKLly10a* and *UAKLla1*) amplifying polymorphic alleles in both parents also followed Mendelian expected frequencies in F_1 embryo and megagametophyte tissues. The results of transmission of these species-specific markers to F_1 progenies are presented in Fig. 2, band C. In crosses between alpine larch (female parent) and western larch (male parent), all embryos carry both the paternal and maternal allele variants consistent with a biparental mode of inheritance (Fig. 2B and 2C). As expected, all the megagametophyte tissues analyzed exhibited only the maternal variant.

Discussion

This study reports on the development of SSR genetic markers for larches. These hypervariable markers are preferred (Hughes and Queller 1993), especially in species with little variation such as western larch (Fins and Seeb 1986) and patched alpine larch (Arno 1990). Attempts to detect losses of variation in isolated patches or to make comparisons of genetic variability between populations would be enhanced through the use of more variable loci. As opposed to other conifers such as pine (Powell et al. 1995; Echt et al. 1996; Vendramin et al. 1996), fir (Vendramin and Ziegenhagen 1997), and spruce (Van de Ven and McNicol 1996; Pfeiffer et al. 1997) for which SSR sequences are available, this is the first report on development of SSRs in larch, and the number of complete sequences in molecular databases is scanty. The initial attempts to isolate SSR markers with a non-enriched library with an average insert size of 500 bp resulted in only one positive clone. This frequency of recovery much lower than expected, was apparently due to the fact that most *Sau*3AI fragments carrying $(AC)_n$ sites exist in the 1–6 kbp range (Smith and Devey 1994; Echt et al. 1996) which were not in the 300–700 bp size selected fragments used for clon-

Fig. 1. (A) Screening of western larch (W.L.) and larch (A.L.), 2 populations each showing the polymorphism levels at locus *UAKLly7*. (B) Identification of heterozygous trees by analysis of PCR amplification products of DNA templates from megagametophyte tissues at locus *UAKLly6*: pattern of segregating alleles for trees #15, #20, and #23; and homozygous tree # 24. M and C are a 20-bp ladder-DNA sizing marker and a control reaction, respectively.

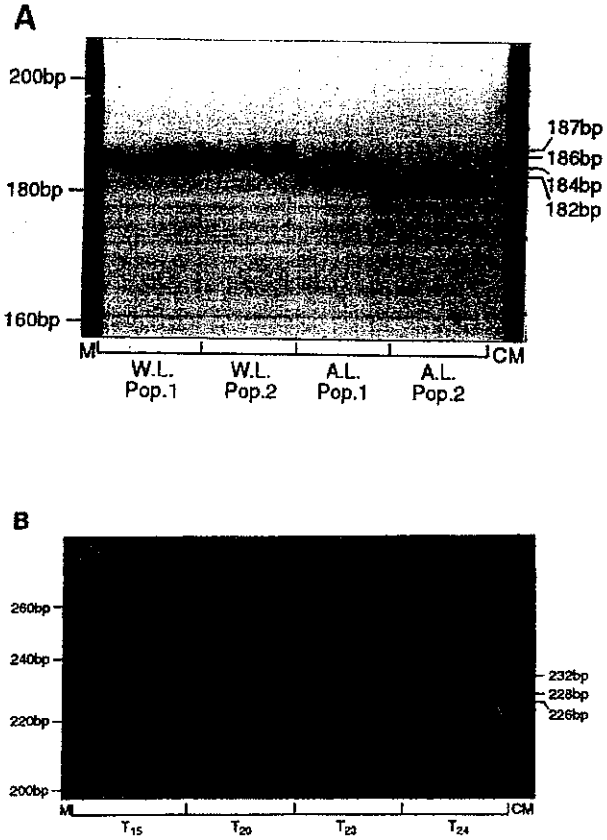
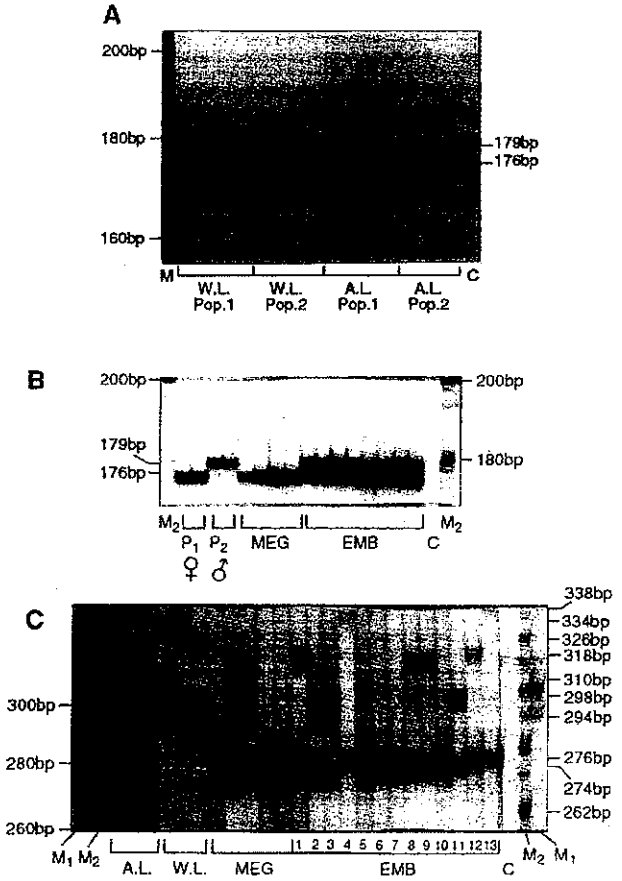


Fig. 2. (A) Pattern of species-specific SSR alleles (*UAKLla1* locus) between western larch (W.L.) and alpine larch (A.L.). (B) Pattern of inheritance of the species-specific SSR alleles from *UAKLla1* locus in F_1 hybrids recovered from crosses between A.L. (P_1, female parent) and W.L. (P_2, male parent). Each of the F_1 embryos (EMB) demonstrated a combination of the A.L., and W.L. species-specific SSR alleles while the maternal allele contribution was present in the megagametophyte tissues (MEG). (C) Pattern of the species-specific SSR locus (*UAKLly10a* locus) for alpine larch (A.L.) and western larch (W.L). Each of the F_1 embryos demonstrated a combination of the A.L., and W.L. species-specific SSR alleles between the two loci. C is the control reaction. M_1 and M_2 are 100-bp and 20-bp ladder-DNA sizing markers, respectively.



ing. Poor recovery of positive clones from the conifer genome has also been attributed to the comparatively low genomic density of these sites and association of the sequence with repetitive DNA (Smith and Devey 1994; Echt et al. 1996). The number of positive clones also depends on the relative proportion of any target SSR in the genome being studied. Libraries constructed from plants by cloning size-selected restriction fragments yield between 10 and 24 positive dinucleotide clones per 10^4 clones screened and about one-tenth this number for tri- and tetranucleotide repeats, though such results depend on the type of SSR sequence targeted and the plant genus (see Kijas et al. 1994). Also, prescreening the SSR positive clones by hybridization to the total labelled genomic DNA prior to their sequencing and primer design is likely to improve the proportion of useful primer pairs identified (Smith and Devey 1994; Pfeiffer et al. 1997). Enrichment of genomic libraries for single- or low-copy sequences also improves the success rate. Indeed, in our case the enriched library had more clones containing at least one defined SSR sequence corroborating reports by Kijas et al. (1994) and Edwards et al. (1996). These results seem to be in good agreement with those of Echt et al. (1996) who found a 200-fold increase in the frequency of $(AC)_n$ clones in an enriched library as compared to a non-enriched library in the pine genome. The enrichment method is therefore recommended, especially for conifer species, to rapidly produce SSR sequences with lower copy number repeats (e.g., tri- and tetranucleotides). Our study showed that most positive clones isolated from the enriched library yielded codominant and informative SSR markers suitable for use in molecular population genetics studies, supporting results by White and Powell (1997). Using a non-enriched library in spruce, only a relatively small proportion of primer pairs (20%) amplified a single variable locus (Pfeiffer et al. 1997).

Table 3. Segregation of microsatellite alleles from megagametophytes of heterozygous mother trees (+), and segregation of species specific alleles in embryos (++) of alpine larch and *G* tests for goodness-of-fit to the 1:1 ratio and heterogeneity among the families.

Locus (size range)	Family	Allelic combination (bp)	Observed segregation	G_{adj}	G_H	G_P	G_T
UAKLLY6+	T11	230/228	10:7	0.51699			
(224–234 bp)	T15	232/226	16:9	1.94750			
	T19	234/226	7:4	0.79261			
	T20	228/224	9:7	0.24306			
	T23	232/226	9:7	0.24306			
	Total				1.85253	0.99803	2.85056
UAKLLY10a+	T11	278/276	7:10	0.51699			
(276–278 bp)	T15	278/276	15:14	0.03391			
	T20	278/276	7:9	0.24306			
	Total				0.55910	0.12912	0.68822
UAKLLY10b+	T11	122/118	6:10	0.98181			
(116–122 bp)	T15	118/116	14:13	0.03632			
	T19	118/116	4:6	0.38520			
	T20	118/116	9:8	0.05707			
	T23	118/116	8:7	0.06469			
	Total				1.47011	0.05295	1.52306
UAKLLY13+	T23	164/160	5:6	0.08708			
(160–164 bp)	T24	164/160	8:6	0.27681			
	Total				0.33772	0.02001	0.35722
UAKLLY14+	T11	250/242	6:9	0.58681			
(216–250 bp)	T15	250/242	17:13	0.52586			
	T19	250/218	6:3	0.98850			
	T20	244/218	5:9	1.10855			
	T23	250/218	9:5	1.11897			
	T24	242/216	7:4	0.80496			
	Total				4.77752	0.26369	5.04121
UAKLLA1++	T11	180/176	20:12	1.96362			
(176–180 bp)	T15	180/176	33:22	2.17737			
	T19	180/176	13:12	0.03880			
	T20	180/176	19:19	0			
	T23	180/176	15:14	0.03330			
	T24	180/176	11:9	0.19461			
	Total				1.84687	1.33212	3.17899

Note: G_{adj} is the *G* test adjusted by Williams' correction; G_T is obtained by adding the *G* for heterogeneity (G_H) and the pooled *G* (G_P). Probability levels of *G*-values > 0.05.

As observed in other organisms, dinucleotide repeats are very much more abundant than tri-or tetranucleotide repeats (Morgante and Olivieri 1993; Smith and Devey 1994; Paetkau and Strobeck 1994; Echt and May-Marquardt 1997). In general and more specifically in animals, the longest run of uninterrupted simple sequence repeats (i.e. minimum number of 10 repeats) is found to be the best predictor of informativeness (Webber 1990). Indeed, Goldstein and Clark (1995) have reported positive correlation between the mean number of repeats and the number of alleles or variance in repeat counts, suggesting that the mutation rate indeed increases with the number of repeats. However, in plants, a shorter run of uninterrupted simple sequence repeats can also be informative even though less polymorphic (Lagercrantz et al. 1993; Terauchi and Konuma 1994). Compound and imperfect dinucleotide repeats are common for larch. Similar observations were also reported for other conifers such as pines (Smith and Devey 1994; Echt et al. 1996; Echt and May-Marquardt 1997). The advantages of tri- and tetranucleotide repeat SSRs are that they are easier to score in manual and automated systems because of a lower incidence of stutter bands and larger unit allelic size differences (Kijas et al. 1995; Scheffield et al. 1995; Echt and May-Marquardt 1997).

Le Page and Basinger (1995) proposed the phylogeny for *Larix* and divided the genus into two morphologically distinct groups: those with bracts of the female cone that are nonexserted and those with bracts exserted. The phylogeny based on allozyme markers (Semerikov and Lascoux 1999), has shown a clear separation between Eurasian and American *Larix* species. Western larch and alpine larch belonging to the *multiseriales* section (bracts exserted) and indigenous to North America are considered closely related species as revealed by the RFLP analysis of cpDNA (Qian et al. 1995). Indeed, research in the U.S.A. has also shown that western larch and alpine larch do form putative natural hybrids in the zones of range overlap (Carlson et al. 1990; Carlson and Theroux 1993) and thus could be considered as sister species or subspecies. Based on the allele numbers, our preliminary observations show that alpine larch is genetically

depauperate compared to western larch. In contrast to Fitzsimmons et al. (1995), who found that the polymorphism levels is higher in species from which the primers were developed, we have found that the polymorphism levels of most SSR loci isolated from alpine larch are higher in its closely related western larch (see also Dayanandan et al. 1997). We also have demonstrated in this study that SSR loci in larch are stable, hypervariable in length, codominant, and are inherited in a simple Mendelian fashion. Some loci (e.g., *UAKLly9*) showed non-amplifying alleles, suggesting the presence of null alleles (Gullberg et al. 1997), but these were not taken into account in this study. The SSR markers isolated from alpine larch successfully amplified SSR loci in western larch and other larch species belonging to both the *pauciseriales* and *multiseriales* sections. Our findings are congruent with other investigators (Thomas and Scott 1993; Powell et al. 1995; Jarne and Lagoda 1996; Dayanandan et al. 1997; Gullberg et al. 1997; Vendramin and Ziegenhagen 1997), who observed conservation of SSR loci and suggest cross-taxa applicability of some SSRs, at least at the subgenus level, as a threshold genetic distance. However, mutation rates for homologous SSR loci may differ not only between taxa, but also between alleles, which certainly complicates the analysis when SSR variation is used to reconstruct historical population events. The potential for the transfer of SSR markers among closely related taxa suggests that considerable time and resources might be saved when primers or molecular databases are available from one of them.

We have found in a population survey study (unpublished data) that the SSR primers developed in this study are sufficiently polymorphic to be used in population genetics and (or) breeding studies for alpine larch and western larch. We found one perfect trinucleotide with five repeats (*UAKLla1* locus) and one imperfect dinucleotide with 2 runs of uninterrupted CA repeat sequences separated by no more than three consecutive non-repeat bases (*UAKLly10a* locus) to be species-specific SSRs for the closely related alpine larch and western larch. They are therefore important for analysis of paternity and studies of introgressive hybridization and microevolutionary processes. The practical significance of these results are to: (*i*) rapidly and easily identify seed lots hybrids (forensics) at any stage in the sporophyte phase of the life cycle in operational nursery practices; (*ii*) depict the patterns of population demography and population differentiation related to taxon delineation, mating system, gene flow, and introgressive hybridization; (*iii*) determine paternity in each population by the probability of excluding an incorrectly assigned father (or mother) and population dynamics; and (*iv*) facilitate a program in genetic improvement (e.g., marker-aided selection) and gene management of larch genetic resources.

Acknowledgements

We are grateful to Drs. R. Hodgetts (Dept. of Biological Sciences, University of Alberta) and Craig Echt (North Central Forest Experiment Station, Forest Service Research, USDA, Rhinelander, Wis.) for their valuable comments on previous drafts of this manuscript. Financial support provided by Forest Renewal BC (FRBC Reference: HQ96090), Natural Sciences and Engineering Research Council of Canada (NSERC, Grant A0342), STEP (Summer Temporary Employment Program) and SCP (Summer Career Placements) programs is gratefully acknowledged. Mr. Leon Theroux (Rocky Mountain Research Station, Forestry Sciences Laboratory, Missoula, Mont.) provided controlled crossed materials and foliage samples of alpine larch parent trees collected from Carlton Ridge. We are also grateful to M.M. Stephen Pluhar, Shahid Nadeem, and Mrs. Mary Aleksuik for their technical assistance.

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Plant Molecular Biology Reporter **19:** 209–215, 2001

Commentary

Nonanchored Inter Simple Sequence Repeat (ISSR) Markers: Reproducible and Specific Tools for Genome Fingerprinting

B. BORNET* and M. BRANCHARD
Laboratoire de Biotechnologie et Physiologie Végétales; ISAMOR-Université de Bretagne Occidentale; F-29280 PLOUZANE, France

Abstract. Many molecular marker techniques are available today. PCR-based approaches are in demand because of their simplicity and requirement for only small quantities of sample DNA. Nonanchored inter simple sequence repeats (ISSRs) are arbitrary multiloci markers produced by PCR amplification with a microsatellite primer. They are advantageous because no prior genomic information is required for their use. We found the technique stable across a wide range of PCR parameters. Polymorphisms were abundant among 7 dicot species tested with 2 tri-nucleotide and 2 tetra-nucleotide primers. Thus, nonanchored ISSR markers are a good choice for DNA fingerprinting.

Key words: molecular markers, nonanchored primer, reproducibility, specificity

Abbreviations: amplified fragment length polymorphisms, AFLPs; cetyltrimethylammonium bromide, CTAB; inter simple sequence repeat, ISSR; microsatellite-primed PCR, MP-PCR; polymerase chain reaction, PCR; random amplified polymorphic DNA, RAPD; simple sequence repeats, SSRs.

Introduction

Since the mid 1980s, genome identification and selection has progressed rapidly with the help of PCR technology. A large number of marker protocols that are rapid and require only small quantities of DNA have been developed. Three widely-used PCR-based markers are RAPDs (Williams et al., 1990), SSRs or microsatellites (Tautz, 1989), and AFLPs (Vos et al., 1995). Each marker technique has its own advantages and disadvantages. RAPD markers are very quick and easy to develop (because of the arbitrary sequence of the primers) but lack reproducibility (Karp et al., 1997; Hansen et al., 1998; Jones et al., 1999; Virk et al., 2000). AFLP has medium reproducibility but is labour intensive and has high operational and development costs (Karp et al., 1997). Microsatellites are specific and highly polymorphous (Karp et al., 1997, Jones et al., 1999), but they require knowledge of the genomic sequence to design specific primers and, thus, are limited primarily to economically important species.

*Author for correspondence. e-mail: benjamin.bornet@univ-brest.fr; fax: 33 2 9805 6101; ph: 33 2 9805 6138.

The choice of a molecular marker technique depends on its reproducibility and simplicity. The best markers for genome mapping, marker assisted selection, phylogenic studies, and crop conservation have low cost and labour requirements and high reliability. Since 1994, a new molecular marker technique called inter simple sequence repeat (ISSR) has been available (Zietkiewicz et al., 1994). ISSRs are semiarbitrary markers amplified by PCR in the presence of one primer complementary to a target microsatellite. Amplification in the presence of nonanchored primers also has been called microsatellite-primed PCR, or MP-PCR, (Meyer et al., 1993). Such amplification does not require genome sequence information and leads to multilocus and highly polymorphous patterns (Zietkiewicz et al., 1994; Tsumara et al., 1996; Nagaoka et al., 1997). Each band corresponds to a DNA sequence delimited by two inverted microsatellites. Like RAPDs, ISSRs markers are quick and easy to handle, but they seem to have the reproducibility of SSR markers because of the longer length of their primers. We now report the effect of various PCR parameters on the specificity and reproducibility of nonanchored ISSR amplifications.

Material and Methods

Sample materials

The following species were used:

- Cauliflower doubled haploid (DH) lines I–VI (*Brassica oleracea* var. *botrytis* L.)
- *Brassica carinata*
- *Arabidopsis thaliana*
- *Phaseolus vulgaris*
- *Helianthus annuus*
- *Solanum tuberosum*
- *Nicotiana tabacum*
- *Homo sapiens*

DNA extraction

Plant genomic DNA was extracted by a CTAB (cetyltrimethylammonium bromide) protocol. Leaf tissues (100 mg) were ground in 1000 µL of CTAB extraction buffer (100 mM Tris [pH 8.0], 1.4 M NaCl, 20 mM EDTA [pH 8.0], 0.2% (p/v) β-mercaptoethanol, 2% [p/v] CTAB) and heated at 60°C for 30 min. DNA was extracted with one volume of a chloroform:isoamyl alcohol mix (24:1) and precipitated in presence of isopropanol (40% [v/v] final concentration). The DNA pellet was washed with 5 mM ammonium acetate and 70% ethanol, dried, and dissolved in 100 µL of TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0]). After addition of 1 µL of RNase (10 mg/mL), DNA concentrations were determined with a fluorometer (Hoefer TKO 100) using bisbenzimide (Hoechst dye 33258) as the fluorescent dye.

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PCR conditions

Four nonanchored oligonucleotide primers, $(CAG)_5$, $(CAA)_5$, $(GACA)_4$, and $(GATA)_4$, were used for amplifications. To optimize the reaction conditions, we tested several PCR parameters, including DNA concentration (0.5-150 ng/reaction, 14 values), primer concentration (10-500 pmol/reaction, 9 values), $MgCl_2$ concentration (0-10 mM, 11 values), dNTP concentration (20-700 mM each, 8 values), Goldstar red DNA polymerase (0.5-2 units/reaction, 5 values), and number of cycles (15-40, 11 values). Reactions without DNA were used as negative controls. The optimum annealing temperature was determined for each primer from a minimum of 5 temperatures. Amplifications were carried out in 3 thermocyclers: GeneAmp PCR system 2400 (Perkin Elmer), Thermoline (Amplitron), and Robot cycler (Stratagene). Amplification conditions were 1 min initial denaturation step (94°C), followed by 27 cycles of 1 min (94°C), 1 min (specific annealing temperature), and 4 min (72°C). The reactions were completed by a final extension step of 7 min (72°C).

Electrophoresis and analysis of amplification products

The PCR products were analyzed by electrophoresis using a 1.5% agarose gel. Once the PCR mix conditions were selected, we also tested various percentages of agarose (0.8, 1, 1.5, 2, and 3%). DNA was stained by soaking the gel in a 0.5 μg/mL ethidium bromide solution.

Results and Discussion

Influences of PCR parameters on nonanchored ISSR amplifications

We investigated several parameters that could affect pattern quality and reproducibility of ISSR fingerprints. First, we analyzed the effect of annealing temperatures (Figure 1). These were estimated based on the Wallace rule for oligonucleotide hybridization. More than 5 temperatures (Figures 1a-1c) were tested for each primer. Using high stringency (Ta higher than Tm), clear and reproducible bands were observed until the PCR reaction was inhibited by high stringency. At lower temperatures, prominent smears appeared that could be eliminated by reducing the loading volume of PCR product. These smears were not caused by nonspecific amplifications but reflected the high reaction yield (data not shown). Temperatures were chosen for each primer that maximized the pattern information (maximum amplification and well-separated bands). Optimal hybridization temperatures were 42°C for $(GATA)_4$ primer, 52°C for $(GACA)_4$ primer, 62°C for $(CAG)_5$ primer, and 54°C for $(CAA)_5$ primer; these temperatures were 2, 4, 12, and 14°C higher than the Wallace temperature, respectively.

Modifications of annealing temperature are known to have a great impact on the richness and legibility of fingerprints. The above pattern variations were likely caused by the greater efficiency of primer hybridization and not to nonspecific hybridization. In many papers, unique and low annealing temperatures have been used for ISSR amplifications with different primers (e.g., Sanchez de la Hoz et al., 1996). However, a low annealing temperature may increase nonspecific amplification, leading to artifact bands. This is reminiscent of the reproducibility

problems with RAPD fingerprints (Hansen et al., 1998). In our case, annealing temperatures were primer-specific and always higher than the Tm. We observed complete and clear patterns in the presence of nonanchored dinucleotide primers (unpublished results). By contrast, Sharma et al. (1995) and Gupta et al. (2000) obtained smears.

Figures 1d-1f show the effect of additional PCR parameters on nonanchored ISSR patterns. Three kinds of patterns were observed: (1) a few discrete bands, (2) a large number of separate bands, and (3) smears. Fingerprints with only a few discrete bands resembled those obtained using limiting quantities of PCR components. In all these cases, the bands corresponded to the most intensive bands in the other patterns and to low or high molecular weight DNA fragments. Smears occurred in the presence of the highest amount of each product when DNA amplification produced a high yield and, again, were not from nonspecific amplifications. Reduction of the electrophoresis loading volume produced the same complete and clear pattern as that observed with the optimal values (data not shown).

The above results indicate that reliable nonanchored ISSR-PCR can be achieved over a broad range of experimental conditions. Variation in primer concentration is one of the main sources of RAPD pattern variations (Hansen et al., 1998; Virk et al., 2000). Our results show no modification of patterns when primer concentrations are changed. Concentrations lower than 50 pmole per reaction produced complete fingerprints, even if the global band intensity was low. Intense and homogeneous banding was observed between 50 and 200 pmole per reaction. Smears were noticed with 500 pmole per reaction – especially with high molecular bands.

The best patterns were amplified in the presence of 12 ng genomic DNA, 100 pmol of primer, 2.5 mM of $MgCl_2$, 200 µM of each of the 4 dNTPs, 1.25 units of Goldstar red DNA polymerase (Eurogentec, Belgium), 1x enzyme buffer, and 27 PCR cycles (as described in Materials and Methods).

Replicate nonanchored ISSR amplifications using 3 different thermocyclers produced the same banding patterns on agarose gels. The best resolving power was obtained with 2% agarose. This produced profiles from the 6 closely related cauliflower lines with 7-12 clear, independent, and high-intensity bands ranging from 150-2500 bp, depending on the primer.

Nonanchored ISSR amplification reproducibility and polymorphism

Using the above amplification protocol, we tested the 4 nonanchored primers for reproducibility within each of the 6 DH lines. Yields and patterns from various concentrations of the hot start (Eurogentec, Belgium) and classical enzymes were identical. The amplifications produced consistent intensity banding profiles for 8 replicate plants within each of the 6 lines for each primer. Typical band patterns from DH I line and $(CAA)_5$ primer are presented in Figure 2, which shows the homogeneity of nonanchored ISSR fingerprints. The amplifications were consistent across DNA samples from the same line and across separate PCR runs. Most of the optimization tests were carried out twice, and identical results were obtained in each case.

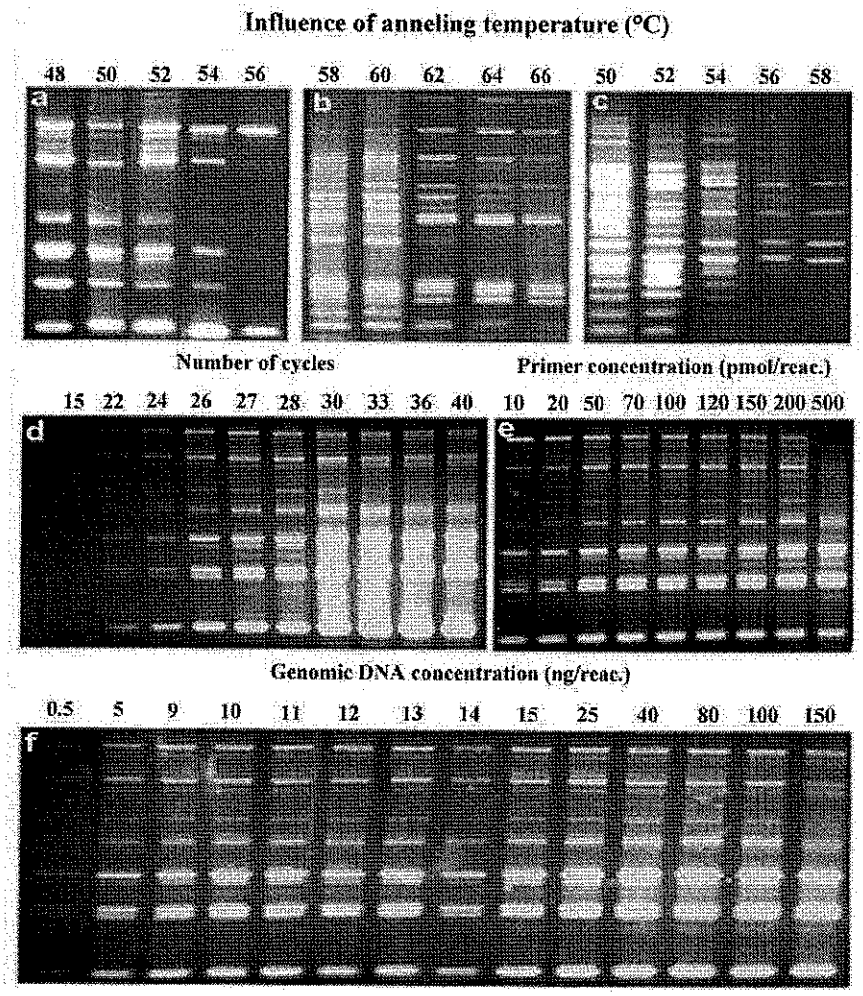

Figure 1. Example of PCR parameter variations on nonanchored ISSR fingerprints. Figures a-c present influence of the annealing temperature upon pattern quality amplified with 3 primers, respectively (a) $(GACA)_4$: 48-56°C, (b) $(CAG)_5$: 58-66°C, and (c) $(CAA)_5$: 50-58°C. The other parameters were tested using the $(GACA)_4$ primer at the optimal annealing temperature: (d) variation of the number of PCR cycles (15-40), (e) variation of the primer concentration (10-500 pmol/reaction), and (f) variation of DNA quantity (0.5-150 ng/reaction).

Comparisons of banding patterns between the 6 closely-related DH lines revealed high polymorphism and complexity. Of the 41 scorable bands, 17 were polymorphous (41.5%). Primers $(CAG)_5$, $(CAA)_5$, and $(GACA)_4$ yielded similar numbers of bands – more than $(GATA)_4$. Poulsen et al. (1994) also found uneven distribution of polymorphisms from the $(GATA)_4$ and $(GACA)_4$ oligonucleotide probes among the 6 *Brassicacea* species forming the "triangle of U". An especially low abundance occurred with *Brassica oleracea* genomes. Banding differences could be found between DH V and VI as well as between DH I, II, III, and IV, although DH IV patterns were the most different. These results are consistent

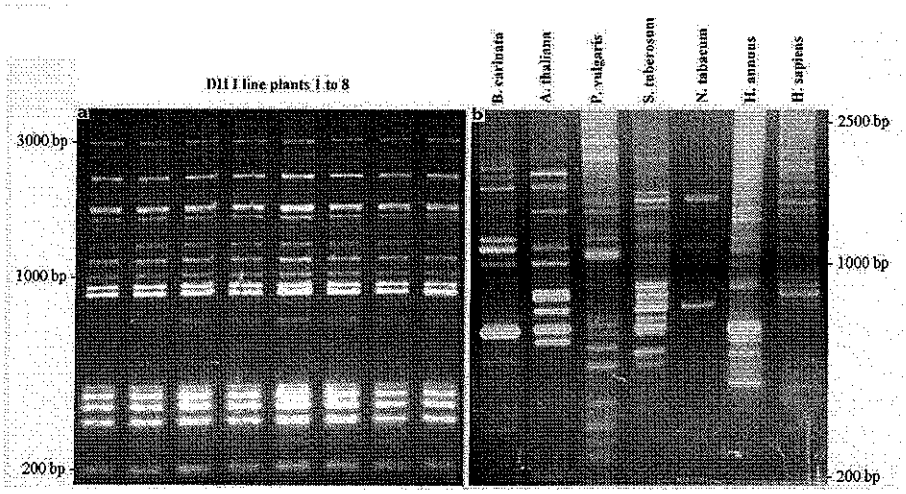

Figure 2. General applicability of nonanchored ISSR markers. (a) Stability of fingerprints from 8 plants of *Brassica oleracea* var. *botrytis* (DH I) amplified by $(CAA)_5$ primers and (b) application of the nonanchored ISSR protocol and $(CAA)_5$ primers reveals polymorphism, between different species and kingdoms, respectively: *Brassica carinata*, *Arabidopsis thaliana*, *Phaseolus vulgaris*, *Solanum tuberosum*, *Nicotiana tabacum*, *Helianthus annuus*, and *Homo sapiens*.

with classifying the 6 DH lines into winter (DH I–IV) and autumn (V and VI) cauliflowers.

To test the general applicability of the nonanchored ISSR protocol, we performed fingerprint amplifications from several additional species (Figure 2b). In all cases, the amplification gave clear, equally complex, and reproducible banding patterns. The bands ranged from 100-2500 bp and exhibited a high level of polymorphism.

In conclusion, nonanchored ISSR markers are universal, quick, easy to apply, highly reproducible, and polymorphous. This is not always the case with the principal alternative, RAPD markers (Karp et al., 1997). Nonanchored ISSR markers will be useful for study of interspecific and intraspecific relationships and in plant breeding.

Acknowledgments

Cauliflower seeds were kindly provided by Dr. Tin Lunn (Organisation Bretonne de Sélection, Plougoulm-France). DNA from human umbilical cord blood cells was kindly provided by Dr. Françoise Arnold (Laboratoire de microbiologie et sécurité alimentaire, ESMISAB, ISAMOR, Technopole Brest-Iroise, Plouzané-France). The authors thank Ms. A. Rawat for reviewing the manuscript. This work was supported by a doctoral fellowship from the Conseil Régional de Bretagne, France.

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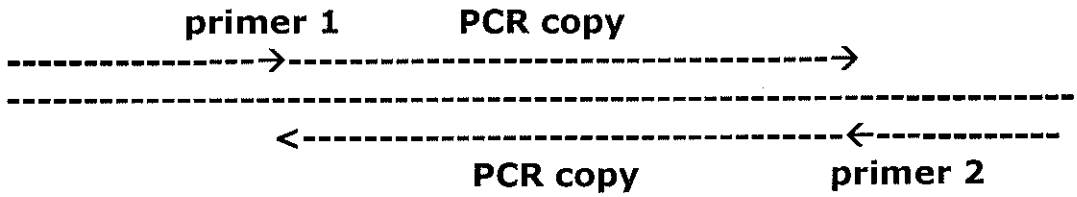
Williams JGK, Kubelik AR, Livak KJ, Rafalski JA and Tingey SV (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res 18: 6231-6235.

Zietkiewicz E, Rafalski A and Labuda D (1994) Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. Genomics 20: 176-183.

When two primers bind in opposite orientations, and reasonably close together, the PCR process can be used copy the DNA sequences between the primers.



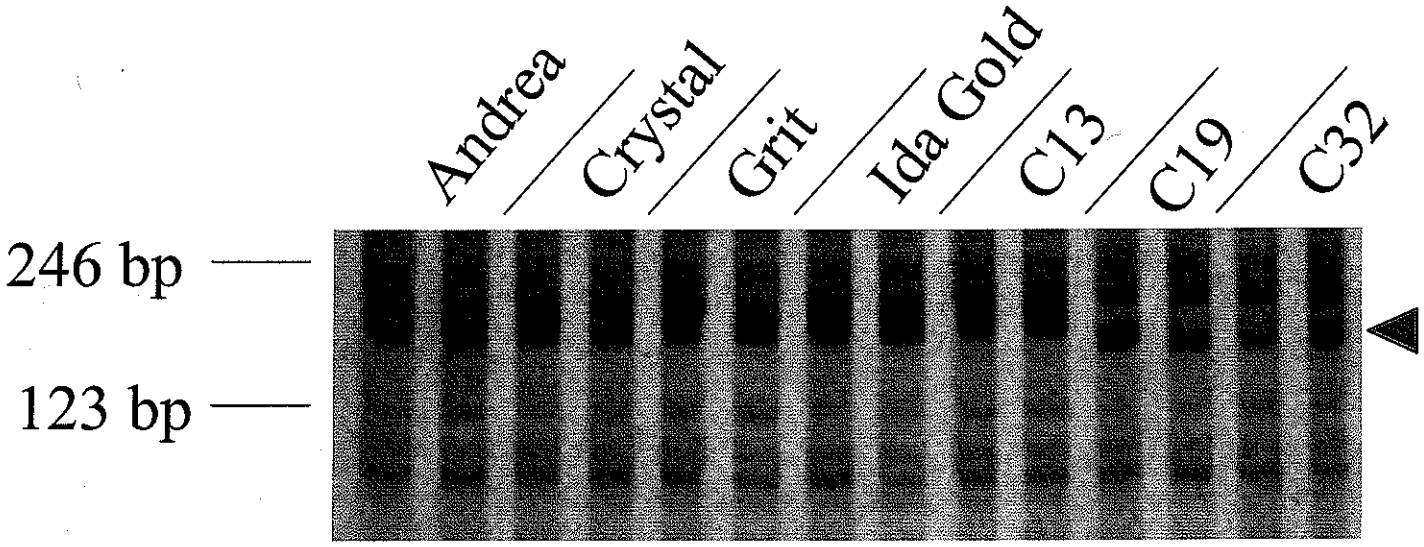
The result is a DNA fragment of a specific length. Typically, this process leads to many PCR products, each with a specific length. This set of DNA fragments serves as a "fingerprint" which can be a unique identifier for a particular crop species and frequently for a given crop variety.

1. Khasa PD, Newton C, Rahman MH, Jaquish B, and Dancik BP (2000) Isolation, characterization, and inheritance of microsatellite loci in alpine larch and western larch. *Genome* 43: 439-448.

2. Bornet B, and Branchard M (2001) Nonanchored Inter simple sequence repeat (ISSR) markers: Reproducible and specific tools for genome fingerprinting. *Plant Molecular Biology Reporter* 19: 209-215.

Cultivar Differentiation in Barley Using ISSR PCR

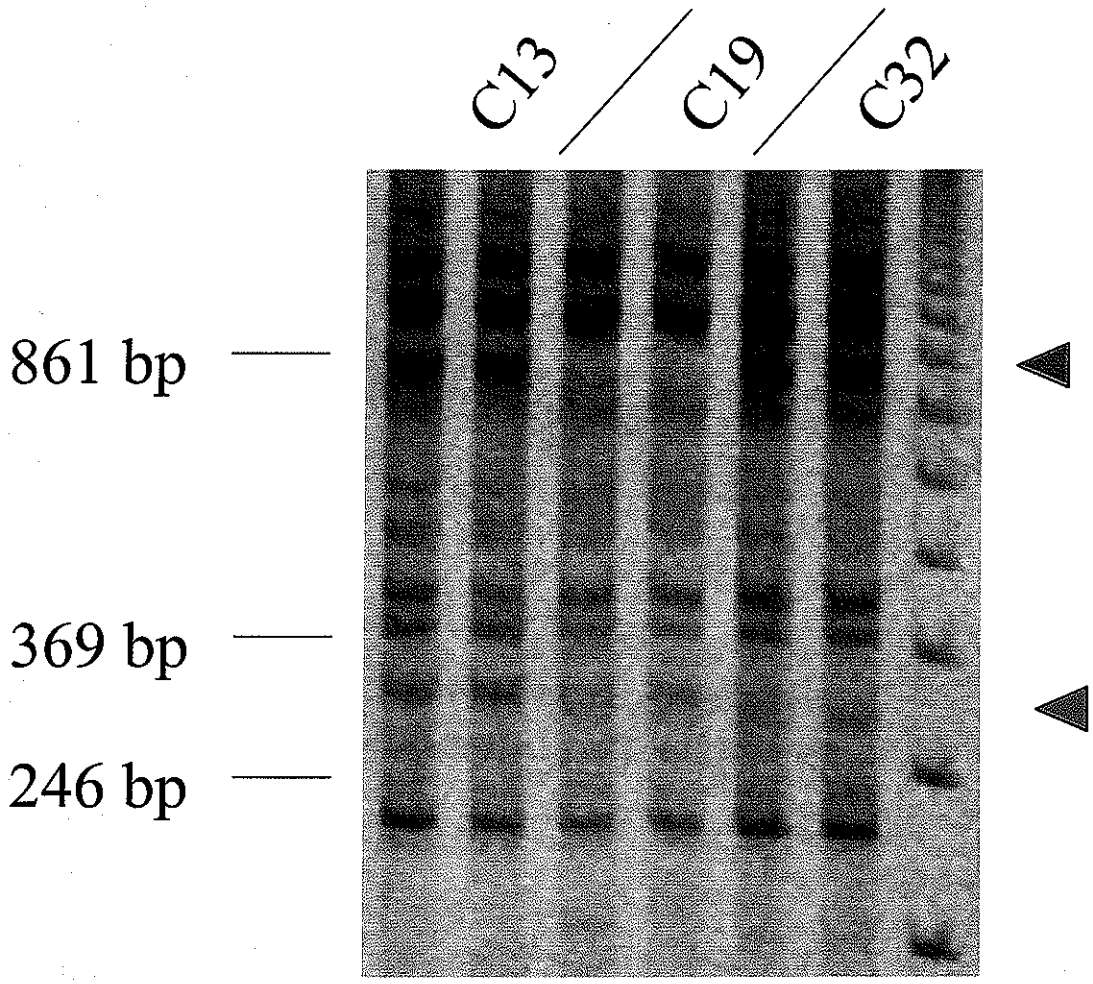
Primer: non-anchored 5x CAG



One PCR product found between 123 bp and 246 bp is slightly smaller in the lines C19 and C32 and can be used to effectively differentiate these two lines from the other five.

Cultivar Differentiation in Barley Using ISSR PCR

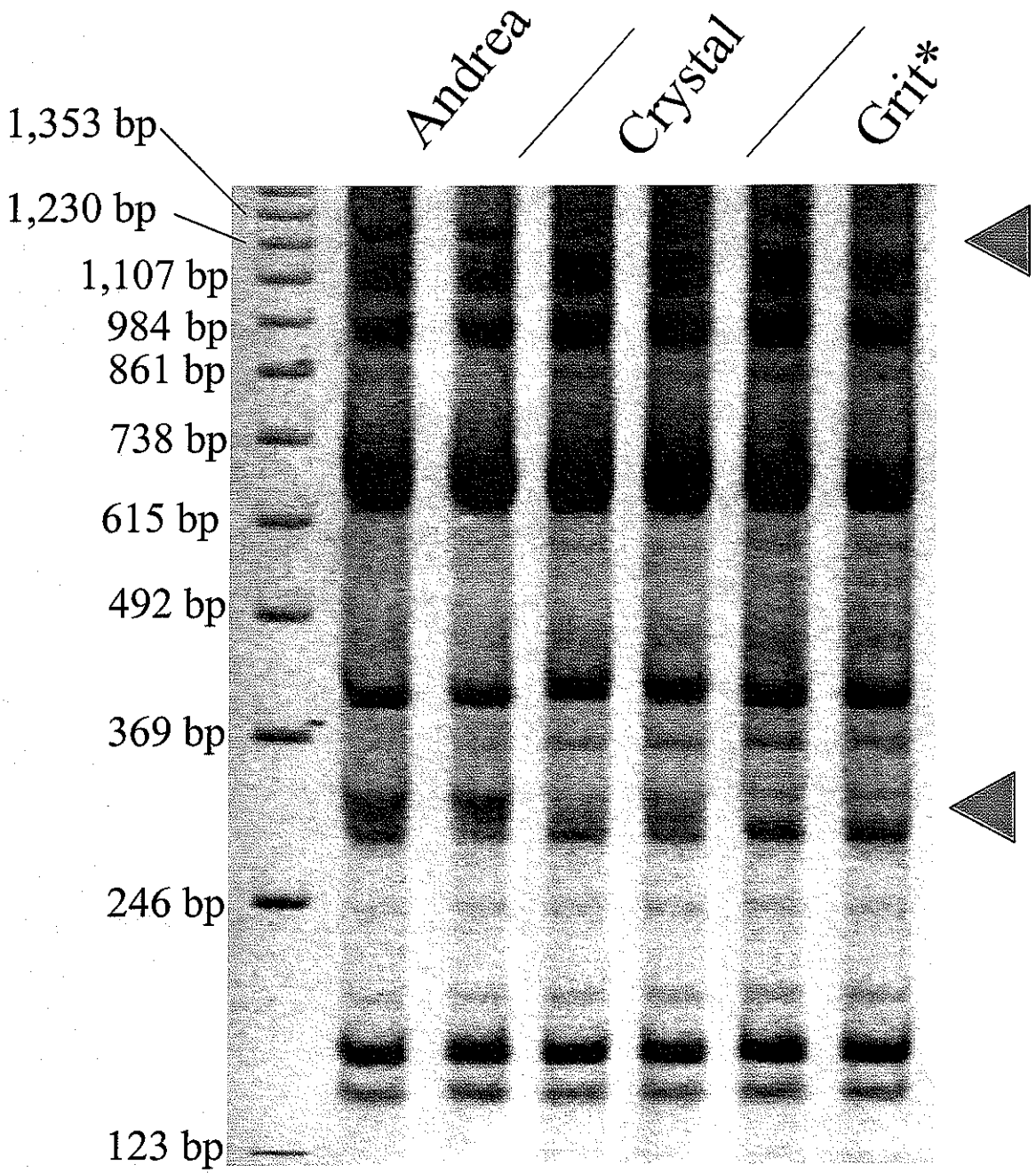
Primer: non-anchored 4X GACA



ATTACHMENT B-5

Cultivar Differentiation in Barley Using ISSR PCR

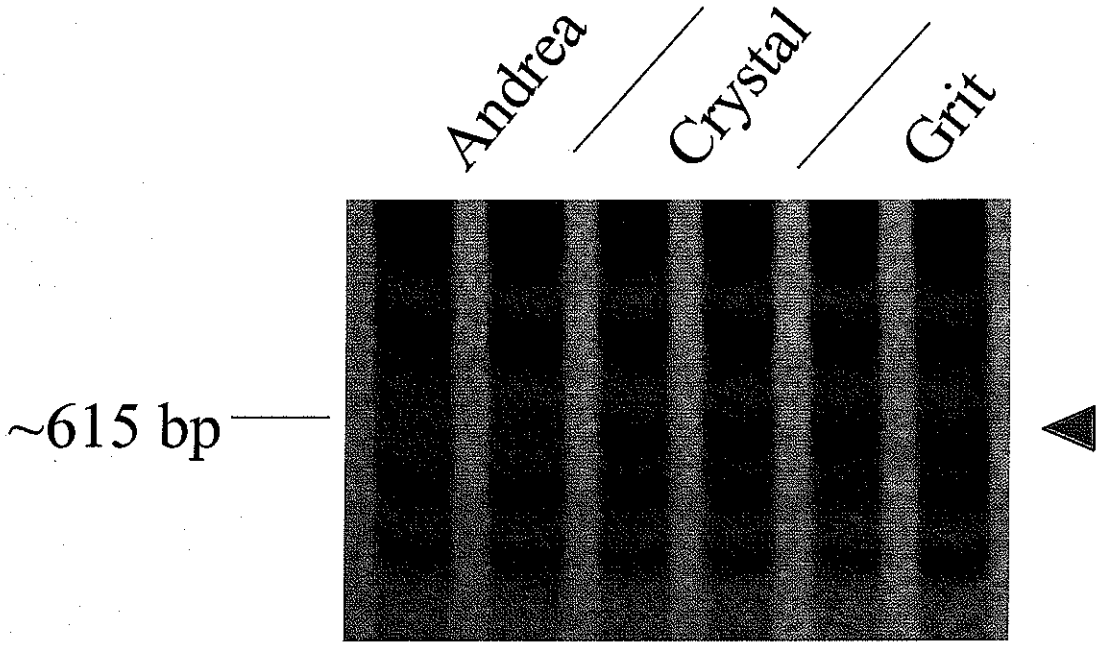
Primer: (TCC)5RY



*C13 is identical to Grit in this test

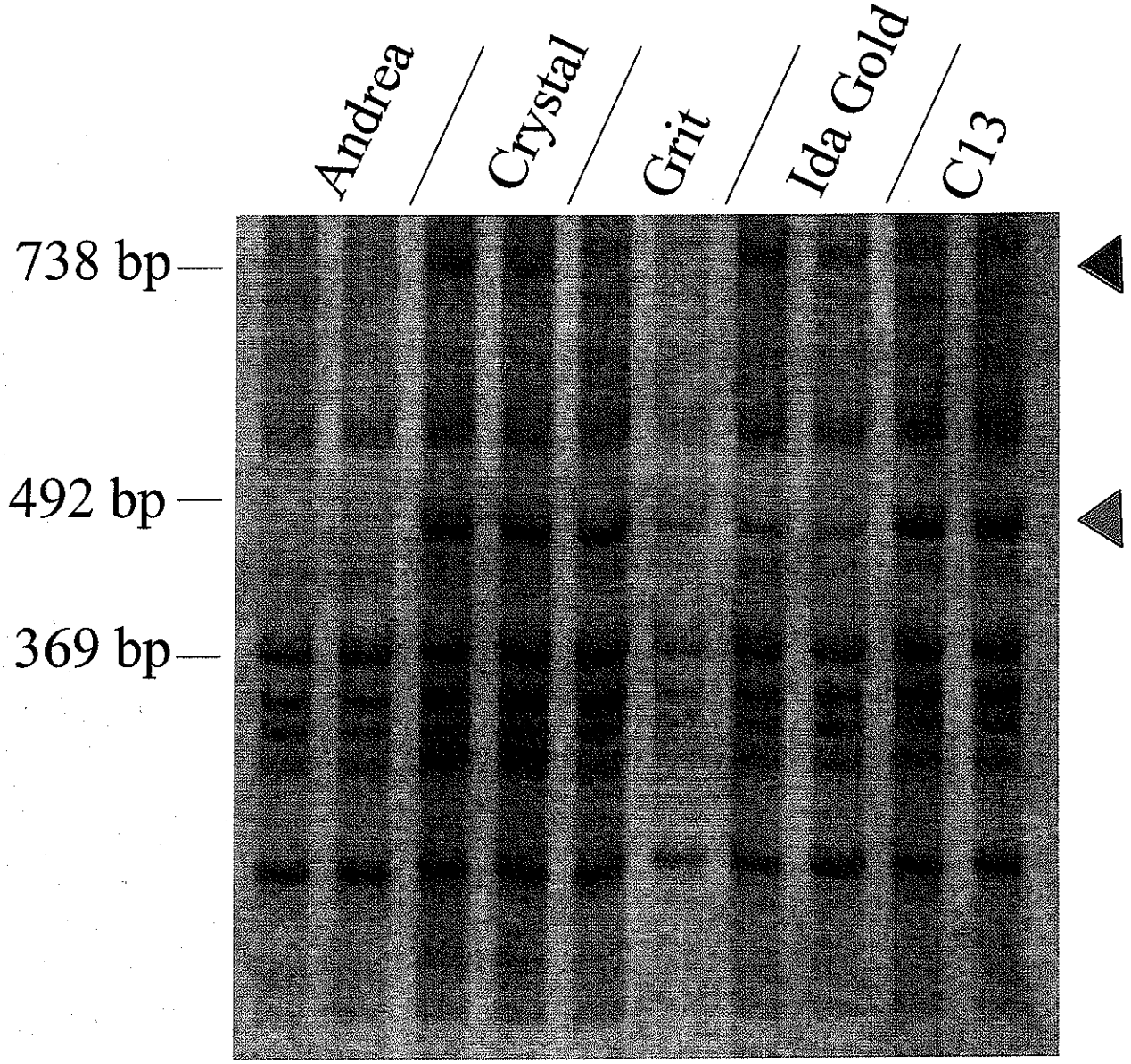
Cultivar Differentiation in Barley Using ISSR PCR

Primer: HVH(TG)7T



Cultivar Differentiation in Barley Using ISSR PCR

Primer: Mixture TCC5RY + HVH(TG)7T



Data Summary

	And	Cry	Grit	Ida	C13	C19	C32
And	x						
Cry	3 615	x					
Grit	2 1,230	2 300	x				
Ida	5 738+	Un-differentiated	Un-differentiated	x			
C13	2 1,230	2 300	Un-differentiated	Un-differentiated	x		
C19	1 200	1 200	1 200	1 200	4 831	x	
C32	1 200	1 200	1 200	1 200	4 831	4 831	x

Primer Key

1. 5X CAG
2. TCC5RY
3. HVH(TG)7T
4. 4X GACA
5. Mix TCC5RY + HVH(TG)7T

The intersecting box represents the information required to differentiate the two cultivars. The top number represents the primer required and the bottom number indicates the approximate size of the "key" DNA band in base pairs (bp).

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1996 SIVPT~Southern Idaho Variety Performance Trial, Burley ID

Variety	Plot Yld.	Test Wt.	bu/ac	lbs/ac @ 12%H2O	lbs/ac over 6/64	% Screen over 6/64	Moisture	Protein	Color	Height	Lodging	Days to Head
C32 (IdaGold II)	11.935	52.95	200.543	10027.2	9497.46	94.75	10.425	11.6025	58.25	27.75	0	77.25
C19	10.83	52.8	182.125	9106.3	8561.08	94	10.325	10.7425	57.25	***31***	0	76.5
IDAGOLD	***10.31***	52.475	***173.251***	***8662.6***	***8054.16***	93	10.425	11.015	59.25	27.5	0	***97***
COV	9.6709	1.0493	9.7077	9.7077	10.1373	1.5369	3.164	10.6019	5.5327	6.9763	*	11.8818
LSD	1.34	0.86	22.70	1135.75	1047.82	2.07	0.55	1.88	5.22	1.88	0.00	0.98

Items in Bold Italics Differ Significantly from IdaGold II (C32)
ANOVA's run with Fisher's Pairwise Comparisons @ 0.05 Individual Error Rate
CV=Coefficient of Variation
LSD=Least Significant Difference

1996 NCVPT~Northern Colorado Variety Performance Trial, Berthoud CO

Variety	Plot Yld.	Test Wt.	bu/ac	lbs/ac @ 12%H2O	lbs/ac over 6/64	% Screen over 6/64	Moisture	Protein	Color	Height	Lodging	Days to Head
C32 (IdaGold II)	8.8	50.725	147.904	7395.2	6346.81	86.25	10.4	9.3143	41	22.75	10	88.5
C19	8.475	48.025	143.195	7159.77	4501.83	63.25	***9.925***	10.6077	34.75	***26.25***	61.25	88.75
IDAGOLD	7.645	***47.5***	129.053	6452.65	***3733.01***	***56.75***	9.975	11.9393	***28.75***	24.5	52.5	***93.75***
CV	10.6239	4.5403	10.5315	10.5315	31.9043	29.1446	3.4037	22.1152	18.9969	10.0734	96.5729	3.1429
LSD	1.21	2.77	20.40	1020.76	1756.662	25.30	0.44	3.47	6.83	3.30	54.22	2.18

Items in Bold Italics Differ Significantly from IdaGold II (C32)
ANOVA's run with Fisher's Pairwise Comparisons @ 0.05 Individual Error Rate
CV=Coefficient of Variation
LSD=Least Significant Difference

1996 SCVPT~Southern Colorado Variety Performance Trial, Center CO

Variety	Plot Yld.	Test Wt.	bu/ac	lbs/ac @ 12%H2O	lbs/ac over 6/64	% Screen over 6/64	Moisture	Protein	Color	Height	Lodging	Days to Head
C32 (IdaGold II)	9.825	48.075	163.949	8197.47	4135.5	47	10.975	15.2069	69.25	26	0	78.5
C19	9.935	49.15	166.455	8322.73	4509.94	54	10.675	14.5358	63.5	***31.5***	***62.5***	78.25
IDAGOLD	8.35	47.1	139.551	6977.56	3401.21	44.75	10.85	15.3727	67.25	25.25	2.5	***80.5***
COV	15.913	5.812	15.709	15.709	53.347	48.003	4.134	10.582	6.958	12.976	148.364	1.658
LSD	2.16	4.46	35.46	1772.56	3508.522	38.58	0.72	2.60	6.57	3.50	18.62	1.32

Items in Bold Italics Differ Significantly from IdaGold II (C32)
ANOVA's run with Fisher's Pairwise Comparisons @ 0.05 Individual Error Rate
CV=Coefficient of Variation
LSD=Least Significant Difference

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2001 PVP~Plant Variety Protection Trial, Burley ID

Variety	Plot Yld.	Test Wt.	bu/ac	lbs/ac @ 12%H2O	lbs/ac over 6/64	% Screen over 6/64	Moisture	Protein	Color	Height	Lodging	Days to Head
IdaGold II	9.13333	53.4333	150.761	7538.07	7140.28	94.6667	12	8.8	56.6667	18	0	77.6667
Andrea	8.49333	***55.0333***	140.467	7023.36	6320.47	90	11.7667	8.63333	***64.3333***	***23.3333***	20	76.3333
C13	8.32667	53.2	136.971	***6848.56***	6552.97	96	12.0667	8.6	54.6667	17	0	78.3333
Crystal	9.14667	54.6667	150.285	7514.26	7262.64	96.6667	12.4	8.73333	***69.6667***	***26***	0	77.6667
Grit	8.72667	51.8667	144.393	7219.65	6200.19	85.6667	11.8	8.73333	***62.6667***	20	26.6667	78.3333
IdaGold	8.52	52.4667	141.577	7078.84	6603.98	93.3333	11.3333	8.8	56.6667	14.3333	0	78.6667
CV	11.86	2.454	11.351	11.351	13.346	6.416	7.81	1.856	9.385	23.174	294.365	1.478
LSD	1.00	0.64	15.82	790.56	809.49	4.55	0.88	0.46	1.82	2.23	20.20	0.86

Items in Bold Italics Differ Significantly from IdaGold II (C32)
ANOVA's run with Fisher's Pairwise Comparisons @ 0.05 Individual Error Rate
CV=Coefficient of Variation
LSD=Least Significant Difference

20030043

FORM APPROVED: OMB NO. 40-R3822

EXHIBIT C
(Barley)

U.S. DEPARTMENT OF AGRICULTURE
AGRICULTURAL MARKETING SERVICE
[redacted]N
BELTSVILLE, MARYLAND 20705

OBJECTIVE DESCRIPTION OF VARIETY
BARLEY *(HORDEUM VULGARE)*

INSTRUCTIONS: *See Reverse.*

NAME OF APPLICANT(S)
Coors Brewing Company

ADDRESS *(Street and No. or R.F.D. No., City, State, and ZIP Code)*
12th and Ford Street, Golden, Colorado 80401
United States of America

FOR OFFICIAL USE ONLY
PVPO NUMBER 200300043
VARIETY NAME OR TEMPORARY DESIGNATION

Place the appropriate number that describes the varietal character of this variety in the boxes below.
Place a zero in first box (i.e. [0][8][9] or [0][9]) when number is either 99 or less or 9 or less.

1. GROWTH HABIT:

[2] 1 = SPRING 2 = FACULTATIVE WINTER 3 = WINTER [2] Early Growth: 1 = PROSTRATE 2 = SEMIPROSTRATE 3 = ERECT

2. MATURITY (50% Flowering):

[2] 1 = EARLY (California Mariout) 2 = MIDSEASON (Betzes) 3 = LATE (Frontier)

[1] No. of days *Earlier* than ..... [9]
[4] No. of days *Later* than ..... [8]
1 = BETZES 2 = CALIFORNIA MARIOUT 3 = CONQUEST 4 = DICKSON 5 = PIROLINE 6 = PRIMUS 7 = UNITAN 8 = Galena 9 = IdaGold

3. PLANT HEIGHT (From soil level to top of head):

[2] 1 = SEMIDWARF 2 = SHORT (California Mariout) 3 = MEDIUM TALL (Betzes) 4 = TALL (Conquest)

[1][2] Cm. *Shorter* than ..... [8]
[0][4] Cm. *Taller* than ..... [9]
1 = BETZES 2 = CALIFORNIA MARIOUT 3 = CONQUEST 4 = DICKSON 5 = PIROLINE 6 = PRIMUS 7 = UNITAN 8 = Galena 9 = IdaGold

4. STEM:

[2] Exertion *(Flag to spike at maturity)*: 1 = 0 - 3 cm. 2 = 3 - 10 cm. 3 = 10 - 15 cm.

[2] Anthocyanin: 1 = ABSENT 2 = PRESENT

[0][4] NO. OF NODES (Originating from node above ground)

[1] Collar Shape: 1 = CLOSED 2 = V-SHAPED 3 = OPEN 4 = MODIFIED CLOSED OR OPEN

[1] Shape of Neck: 1 = STRAIGHT 2 = SNAKY 3 = OTHER (Specify) _____

5. LEAF:

[1] Basal leaf sheath *(seedling)*: 1 = GLABROUS 2 = PUBESCENT

[2] Position of flag leaf *(at boot stage)*: 1 = DROOPING 2 = UPRIGHT

[2] Waxiness: 1 = ABSENT (Glossy) 2 = SLIGHTLY WAXY 3 = WAXY

[1][1] MM. WIDTH (First leaf below flag leaf)

[1][6] CM. LENGTH (First leaf below flag leaf)

[2] Anthocyanin in leaf sheath: 1 = ABSENT 2 = PRESENT

6. HEAD:

[1] Type: 1 = TWO-ROWED 2 = SIX-ROWED

[1] Density: 1 = LAX 2 = ERECT (Not dense) 3 = ERECT (Dense)

[2] Shape: 1 = TAPERING 2 = STRAP 3 = CLAVATE 4 = OTHER (Specify) _____

[1] Waxiness: 1 = ABSENT (Glossy) 2 = SLIGHTLY WAXY 3 = WAXY

[1] Lateral Kernels Overlap: 1 = NONE 2 = AT TIP 3 = 1/4 - 1/2 OF HEAD

[1] Rachis *(Hair on edge)*: 1 = LACKING 2 = FEW 3 = COVERED

7. GLUME:

[2] Length: 1 = 1/3 OF LEMMA 2 = 1/2 OF LEMMA 3 = MORE THAN 1/2 OF LEMMA

[1] Hairs: 1 = NONE 2 = SHORT 3 = LONG

[4] Hair covering: 1 = NONE 2 = RESTRICTED TO MIDDLE 3 = CONFINED TO BAND 4 = COMPLETELY COVERED

[2] Awns: 1 = LESS THAN EQUAL TO LENGTH OF GLUMES 2 = EQUAL TO LENGTH OF GLUMES 3 = MORE THAN EQUAL TO LENGTH OF GLUMES

[3] Awn Surface: 1 = SMOOTH 2 = SEMISMOOTH 3 = ROUGH

8. LEMMA:

5 Awn: 1 = AWNLESS 2 = AWNLETS ON CENTRAL ROWS, AWNLESS ON LATERAL ROWS 3 = SHORT ON CENTRAL ROWS, AWNLETS ON LATERAL ROWS 4 = SHORT (less than equal to length of spike) 5 = LONG (longer than spike) 6 = HOODED

4 Awn Surface: 1 = AWNLESS 2 = SMOOTH 3 = SEMISMOOTH 4 = ROUGH

3 Teeth: 1 = ABSENT 2 = FEW 3 = NUMEROUS

1 Hair: 1 = ABSENT 2 = PRESENT

1 Shape of base: 1 = DEPRESSION 2 = SLIGHT CREASE 3 = TRANSVERSE CREASE

2 Rachilla Hairs: 1 = SHORT 2 = LONG

9. STIGMA:

2 Hairs: 1 = FEW 2 = MANY

10. SEED:

2 Type: 1 = NAKED 2 = COVERED

1 Hairs on Ventral Furrow: 1 = ABSENT 2 = PRESENT

3 Length: 1 = SHORT (8.0 mm.) 2 = SHORT TO MIDLONG (7.5 - 9.0 mm.) 3 = MIDLONG (8.5 - 9.5 mm.) 4 = MIDLONG TO LONG (9.0 - 10.5 mm.) 5 = LONG (10.0 mm.)

4 Wrinkling of hull: 1 = NAKED 2 = SLIGHTLY WRINKLED 3 = SEMIWRINKLED 4 = WRINKLED

1 Aleurone Color: 1 = COLORLESS (White or Yellow) 2 = BLUE

0 0 PERCENT ABORTIVE

5 4 GMS. PER 1000 SEEDS

11. DISEASE: (0 = Not Tested, 1 = Susceptible, 2 = Resistant)

0 SEPTORIA	0 NET BLOTCH	0 SPOT BLOTCH	0 POWDERY MILDEW
0 LOOSE SMUT	0 BACTERIAL BLIGHT	0 COVERED SMUT	0 FALSE LOOSE SMUT
0 STEM RUST	0 LEAF RUST	0 SCAB	0 SCALD
0 AY	0 BSMV	0 BYDV	0 OTHER (Specify)

12. INSECT: (0 = Not tested, 1 = Susceptible, 2 = Resistant)

0 GREEN BUG	0 ENGLISH GRAIN APHID	0 CHINCH BUG	0 ARMYWORM
0 GRASS HOPPERS	0 CEREAL LEAF BEETLE	0 OTHER (Specify)	

HESSIAN FLY RACES: 0 GP 0 A 0 B 0 C; 0 D 0 E 0 F 0 G

13. CHEMICAL (0 = Not Tested, 1 = Susceptible, 2 = Resistant)

0 DDT 0 OTHER (Specify)

14. INDICATE WHICH VARIETY MOST CLOSELY RESEMBLES THAT SUBMITTED:

CHARACTER	NAME OF VARIETY	CHARACTER	NAME OF VARIETY
Plant tillering	IdaGold	Seed size	IdaGold
Leaf size	Crystal	Coleoptile elongation	IdaGold
Leaf color	Crystal	Seedling pigmentation	Crystal
Leaf carriage	IdaGold		

REFERENCES: The following publications may be used as a reference aid for the standardization of character descriptions and terms used in this form:

1. Wiebe, G. A., and D. A. Reid, 1961, Classification of Barley Varieties Grown in the United States and Canada in 1958, Technical Bulletin No. 1224, U.S. Dept. of Agriculture.
2. Reid, D. A., and G. A. Wiebe, 1968, Barley: Origin, Botany, Culture, Winter Hardiness, Genetics, Utilization, Pests, Agriculture Handbook No. 338, U.S. Dept. of Agriculture. pp. 61 - 84.
3. Malting Barley Improvement Association, Milwaukee, Wisconsin, 1971, Barley Variety Dictionary.

COLOR: Nickerson's or any recognized color fan may be used to determine color of the described variety.

U.S. DEPARTMENT OF AGRICULTURE
AGRICULTURAL MARKETING SERVICE

EXHIBIT E
STATEMENT OF THE BASIS OF OWNERSHIP

Application is required in order to determine if a plant variety protection certificate is to be issued *(7 U.S.C. 2421)*. The information is held confidential until the certificate is issued *(7 U.S.C. 2426)*.

1. NAME OF APPLICANT(S)	2. TEMPORARY DESIGNATION OR EXPERIMENTAL NUMBER	3. VARIETY NAME
Coors Brewing Company	C32	IdaGold II

4. ADDRESS *(Street and No., or R.F.D. No., City, State, and ZIP, and Country)*	5. TELEPHONE *(Include area code)*	6. FAX *(Include area code)*
12th and Ford Street Golden, Colorado 80401 United States of America	(303) 279-6565	(303) 277-7373

7. PVPO NUMBER: Not Yet Assigned 200300043

8. Does the applicant own all rights to the variety? Mark an "X" in the appropriate block. **If no, please explain** ☑ YES ☐

9. Is the applicant (individual or company) a U.S. National or a U.S. based company? **If no, give name of country** ☑ YES ☐ NO

10. Is the applicant the original owner? ☑ YES ☐ NO **If no, please answer one of the following:**

a. If the original rights to variety were owned by individual(s), is (are) the original owner(s) a U.S. National(s)?

☐ YES ☐ NO **If no, give name of country**

b. If the original rights to variety were owned by a company(ies), is (are) the original owner(s) a U.S. based company?

☐ YES ☐ NO **If no, give name of country**

11. Additional explanation on ownership *(if needed, use the reverse for extra space)*:

All of the specific individuals listed in Exhibit A (e.g. James M. Jakicic, Roy J. Hanson, Kathy R. Adams, Berry J. Treat, Dennis J. Dolan, James W. Hettinger, and any others which may have been involved in the creation of IdaGold II) were employed by Applicant (Coors Brewing Company) during breeding, development, production, testing, increase, and/or completion of barley variety IdaGold II (originally designated with temporary/experimental number "C32" as noted above). By contract with its employees, Applicant (Coors Brewing Company) is the exclusive owner of the present application and all subject matter covered thereby and recited therein including but not limited to exclusive rights in and to barley variety IdaGold II.

PLEASE NOTE:

Plant variety protection can only be afforded to the owners (not licensees) who meet the following criteria:

1. If the rights to the variety are owned by the original breeder, that person must be a U.S. national, national of a UPOV member country, or national of a country which affords similar protection to nationals of the U.S. for the same genus and species.

2. If the rights to the variety are owned by the company which employed the original breeder(s), the company must be U.S. based, owned by nationals of a UPOV member country, or owned by nationals of a country which affords similar protection to nationals of the U.S. for the same genus and species.

3. If the applicant is an owner who is not the original owner, both the original owner and the applicant must meet one of the above criteria.

The original breeder/owner may be the individual or company who directed the final breeding. See Section 41(a)(2) of the Plant Variety Protection Act for definitions.